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REMARKS/ARGUMENTS

Claims 7-24 are active in this application. Support for these claims is found in Claims 1-6 and the specification as originally filed. No new matter is added by these amendments.

Applicants thank Examiner McKenzie for the courteous discussion granted to the Applicants' undersigned representative on December 17, 2003. During this discussion, various proposed amendments to address the rejections in the Office Action were addressed. The amendments submitted herein are consistent with that discussion.

In addition, the enablement rejection was also discussed as it concerns the ability of the antagonists of PPAR α/γ to treat diseases of diabetic ketoacidosis or obesity (referring to new Claims 15 and 24). The Examiner pointed to the description in Nuss where agonists are known to treat diabetic conditions and therefore agonists, such as those in the present application, would not treat the conditions but would make the conditions worse. However, the undersigned pointed the Examiner's attention to the discussion on page 5 of the application which correlates the expression of the PPAR with the particular disease as indicated. The Examiner suggested providing additional information that correlates the ability to antagonize PPAR α/γ with the conditions being treated. This information is attached and discussed below.

In the first publication of Yamauchi et al ((2001) J. Biol. Chem. 276(44):41245-41254), the results concerning PPARγ deficient mice is described. The data show that inhibition of PPARγ is effective for treating obesity or diseases such as diabetes (see the discussion on page 41253, 3rd paragraph). The second attached publication, Yamauchi et al (2001) J. Clin. Invest. 108(7):1001-1013, describes that "functional antagonism of PPARγ/RXR may be a logical approach to protection against obesity and related diseases such as type 2 diabetes." (see the Abstract, last line, page 1001). In this publication, data of the pharmaceutical activity of bisphenol A diglycidyl ether (BADGGE), which is a PPAR γ

antagonist. The antagonist lowers triglyceride in adipocyte or skeletal muscle, which suggests that obesity and type 2 diabetes can be treated accordingly (se page 1005, second column: "Bother the RXR antagonist HX531 and the PPARγ antagonist BADGE exert antiobesity and antidiabetic effects in part through leptin-dependent pathways." As a result, Claims 15 and 24 are enabled by the specification as originally filed.

Concerning the issues raised in the Office Action:

(1) The title has been amended as suggested by the Examiner;

(2) The rejection of Claims 1-4 under 35 U.S.C. § 112, second paragraph is obviated by the cancellation of Claims 1-4;

(3) The rejection of Claims 1-4 under 35 U.S.C. § 101 is obviated by the cancellation of Claims 1-4;

(4) The rejection of Claims 5 and 6under 35 U.S.C. § 112, second paragraph is obviated by the cancellation of Claims 5 and 6;

(5) The rejection of Claims 5 and 6 under 35 U.S.C. § 112, first paragraph is obviated by the cancellation of Claims 5 and 6;

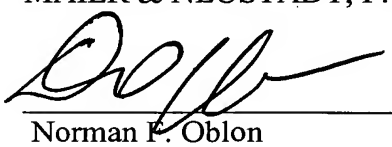
(6) The rejection of Claims 5 under 35 U.S.C. § 102(b) over Nordlie is obviated by the cancellation of Claim 5; and

(7) The rejection of Claims 5 and 6 under 35 U.S.C. § 102(b) based upon publica use or sale of the invention is obviated by the cancellation of Claims 5 and 6.

Applicants request that allowance of this application. Early notice of this allowance is requested.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.



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2

Inhibition of RXR and PPARγ ameli rates diet-induced ob sity and type 2 diab tes

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PPARγ is a ligand-activated transcription factor and functions as a heterodimer with a retinoid X receptor (RXR). Supraphysiological activation of PPARγ by thiazolidinediones can reduce insulin resistance and hyperglycemia in type 2 diabetes, but these drugs can also cause weight gain. Quite unexpectedly, a moderate reduction of PPARγ activity observed in heterozygous PPARγ-deficient mice or the Pro12Ala polymorphism in human PPARγ, has been shown to prevent insulin resistance and obesity induced by a high-fat diet. In this study, we investigated whether functional antagonism toward PPARγ/RXR could be used to treat obesity and type 2 diabetes. We show herein that an RXR antagonist and a PPARγ antagonist decrease triglyceride (TG) content in white adipose tissue, skeletal muscle, and liver. These inhibitors potentiated leptin's effects and increased fatty acid combustion and energy dissipation, thereby ameliorating HF diet-induced obesity and insulin resistance. Paradoxically, treatment of heterozygous PPARγ-deficient mice with an RXR antagonist or a PPARγ antagonist depletes white adipose tissue and markedly decreases leptin levels and energy dissipation, which increases TG content in skeletal muscle and the liver, thereby leading to the re-emergence of insulin resistance. Our data suggested that appropriate functional antagonism of PPARγ/RXR may be a logical approach to protection against obesity and related diseases such as type 2 diabetes.

J. Clin. Invest. 108:1001–1013 (2001). DOI:10.1172/JCI200112864.

Introduction

PPARγ is a ligand-activated transcription factor and a member of the nuclear hormone receptor superfamily that functions as a heterodimer with a retinoid X receptor (RXR) (1–5). Agonist-induced activation of PPARγ/RXR is known to increase insulin sensitivity (6, 7), and thiazolidinediones (TZD), which have the ability to directly bind and activate PPARγ (6) and stimulate adipocyte differentiation (2, 3, 8), are used clinically to reduce insulin resistance and hyperglycemia in type 2 diabetes, though these drugs have been associated with weight gain (9). UK Prospective Diabetes Study has clearly demonstrated that weight gain associated with diabetes treatment partially cancels the beneficial effects of tight blood glucose control on cardiovascular events and mortality (10). Thus, we sought to identify novel therapeutic strategies not only for insulin resistance but also obesity.

We and others have reported that heterozygous PPARγ-deficient mice are protected from high-fat diet–induced (HF diet-induced) or aging-induced adipocyte hypertrophy, obesity, and insulin resistance (11, 12). Consistent with this, the Pro12Ala polymorphism in human PPARγ2, which moderately reduces the transcriptional activity of PPARγ, has been shown to confer resistance to type 2 diabetes (13–15). These

findings raise the following important unresolved issues. First, it remains to be ascertained whether functional antagonism of PPARγ/RXR, e.g., administering an RXR antagonist or a PPARγ antagonist, could indeed serve as an effective treatment strategy for obesity and type 2 diabetes. Second, the mechanism by which reduced PPARγ/RXR activity improves insulin resistance is unclear. Third, whether further reduction of PPARγ/RXR activity is associated with further improvement of insulin resistance, remains to be clarified. To address these issues, we employed pharmacological inhibitors of PPARγ/RXR, a PPARγ antagonist, and an RXR antagonist, in both wild-type and heterozygous PPARγ-deficient mice.

Bisphenol A diglycidyl ether (BADGE) has been reported to act as a relatively selective antagonist for PPARγ (16). In fact, the inhibition of PPARγ transcriptional activity by BADGE was approximately 70%, whereas PPARδ was inhibited by approximately 23% and PPARα was not inhibited. In addition, BADGE was ineffective in attenuating glucocorticoid receptor–mediated transcriptional activation; however, an inhibitory effect of BADGE (~30%) on ligand-induced activation of RXRα was observed. We have recently identified a synthetic RXR antagonist, HX531 (17), and herein show HX531 to be a potential PPARγ/RXR inhibitor in an in vitro transactivation assay and to prevent triglyceride (TG) accumulation in 3T3L1 adipocytes. We also show that administration of the RXR antagonist HX531 or the PPARγ antagonist BADGE to mice on a HF diet decreases TG content in white adipose tissue (WAT), skeletal muscle, and the liver due to increased leptin effects and increased fatty acid combustion and energy dissipation, thereby ameliorating HF diet–induced obesity and insulin resistance, in proportion to their potencies as PPARγ/RXR inhibitors in vitro. Paradoxically, treatment of heterozygous PPARγ-deficient mice with the RXR antagonist or the PPARγ antagonist depletes WAT and markedly decreases leptin levels and energy dissipation, which increases the TG content of skeletal muscle and the liver, thereby causing re-emergence of insulin resistance. Our data suggest that appropriate functional antagonism of PPARγ/RXR may be a logical approach to protection against obesity and related diseases such as type 2 diabetes.

Methods

Chemicals. HX531 (17), rosiglitazone, LG100268 (7), and BADGE (16) were synthesized as described elsewhere. We measured the plasma concentrations of HX531 and BADGE by HPLC (HX531) (17) and gas chromatography (BADGE) (16) in C57 mice orally administered 100 mg/kg of HX531 or 3 g/kg of BADGE. The maximal concentration (C_{max}), the time required until maximal concentration (T_{max}), the area under the plasma concentration time curve (AUC), and the elimination half-life ($t_{1/2}$) for HX531 were 4.1 μg/ml, 1.5 hours, 19.3 μg h/ml, and 1.9 hours, respectively. The C_{max}, T_{max}, AUC, and $t_{1/2}$ for BADGE were 0.45 μg/ml, 9 hours, 8.2 μg h/ml, and 6.4 hours, respectively. Thus, it is reasonable to assume that PPARγ and RXR in animal tissues are exposed to concentrations of compounds essentially comparable to those in in vitro cell culture experiments. Wy-14,643 was purchased from Biomol Research Laboratories (Plymouth Meeting, Pennsylvania, USA). T3, 3-isobutyl-1-methylxanthine, and insulin were from Sigma Chemical Co. (St. Louis, Missouri, USA) 1α, 25-dihydroxy-vitamin D3 was from Calbiochem-Novabiochem Corp. (San Diego, California, USA) Dexamethasone was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other materials were from the sources given in refs. 8, 11, 17, and 18.

Animals, in vivo glucose homeostasis, assay of endogenous serum leptin concentrations, leptin sensitivity, and O_2 consumption. Heterozygous PPARγ-deficient mice have been described previously (11). All other animals were purchased from Nippon CREA Co. Ltd. (Shizuoka, Japan) Six-week-old mice were fed powdered chow according to methods described previously (11). HX531, BADGE, LG100268, or Wy-14,643 was given as a 0.1%, 3%, 0.02%, or 0.01% food admixture, respectively, or an indicated percentage (8, 11), and no toxicity, such as liver damage, was observed. Insulin tolerance tests were carried out according to a method described previously (11), with slight modification. Leptin was assayed with the ELISA-based Quantikine M mouse leptin immunoassay kit (R&D Systems Inc., Minneapolis, Minnesota, USA) according to the manufacturer's instructions. For leptin sensitivity (11), leptin Sigma Chemical Co., St. Louis, Missouri, USA) was administered to mice after treatment with HX531 for 10 days as a daily intraperitoneal injection of 10 μg/g body weight/day. Isotonic sodium chloride solution was administered to the controls. Food intake and body weight were measured to assess the effects of leptin administration. Oxygen consumption was determined as described previously (18). The animal care and procedures were approved by the Animal Care Committee of the University of Tokyo.

Insulin resistance index. The insulin resistance index was calculated from the product of the area of glucose and insulin multiplied by 10^{-3} as described in ref. 7. The areas of glucose and insulin curves were calculated by multiplying the cumulative mean height of the glucose values (1 mg ml^{-1} = 1 cm) and insulin values (1 ng ml^{-1} = 1 cm), respectively, by time (60 min = 1 cm). The results are expressed as the percentage of the value of each control.

Histological analysis of adipose and hepatic tissues and determination of adipocyte size. Adipose tissue was removed from each animal and fixed in 10% formaldehyde/PBS and maintained at 4°C until use. Fixed specimens were dehydrated, embedded in tissue-freezing medium (Tissue-Tek OCT compound; Miles Inc., Elkhart, Indiana, USA) and frozen in dry ice and acetone. WAT was cut into 10-μm sections, and the sections were mounted on silanized slides. The adipose tissue was stained with hematoxylin and eosin (H&E). Mature white adipocytes

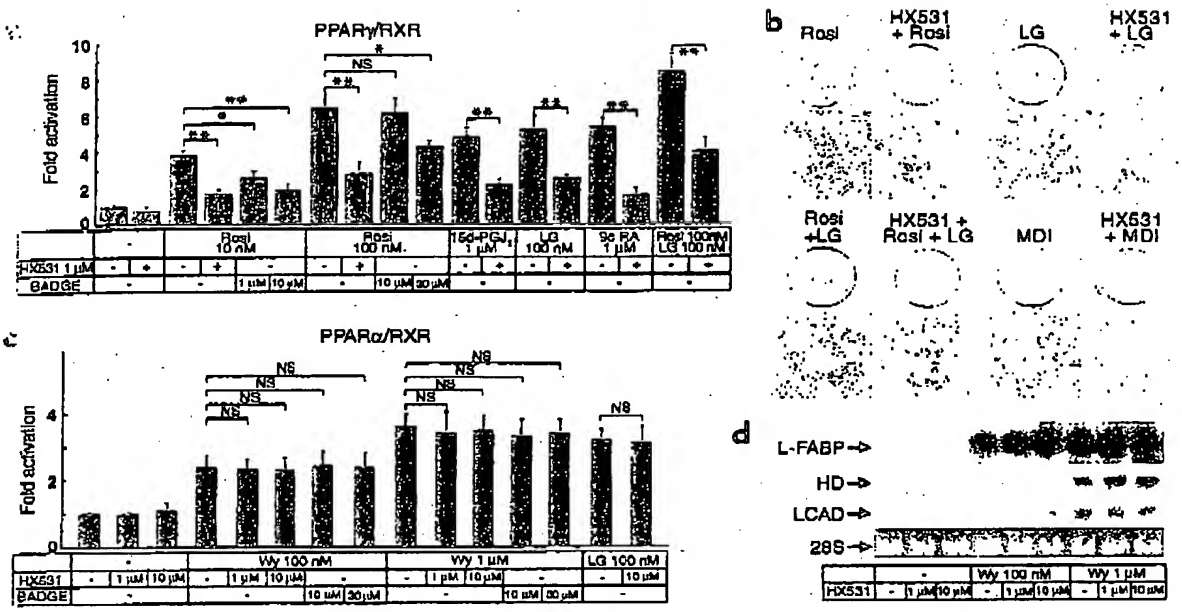

Figure 1

The RXR antagonist HX531 serves as a functional PPARγ/RXR inhibitor (a and c). Transactivation analysis of PPARγ/RXR (a), PPARα/RXR (c). CV-1 cells were cotransfected with RXRα with an expression vector for PPARγ or PPARα. PPARγ/RXR or PPARα/RXR activity was assessed on a PPRE-tk LUC (24) as described previously (22, 23). CV-1 cells were treated with the indicated concentrations of rosiglitazone (Rosi), LG100268 (LG), 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2 (15d-PGJ_2), Wy-14,643 (Wy), BADGE, and HX531. 9-cis-retinoic acid (b) 3T3L1 adipocyte differentiation assay. Oil red O staining for fat accumulation in cells at day 6 after induction. Cells were grown to confluence and then induced to differentiate by exposure to 100 nM rosiglitazone, 1 μM LG100268, or 10 μM HX531, or by conventional hormonal stimuli (MDI; a combination of 3-isobutyl-1-methylxanthine, dexamethasone, and insulin). (d) Amounts of the mRNAs of liver-type fatty acid–binding protein (L-FABP), HD, and long-chain acyl-CoA dehydrogenase (LCAD) in rat hepatoma FAO cells treated with the indicated concentrations of Wy-14,643 and HX531 for 24 hours. Each bar represents the mean ± SE (n = 5–10). $^*P < 0.05$, $^{**}P < 0.01$ with versus without HX531 or BADGE. NS, no significant difference.

were identified by their characteristic multilocular appearance. Total adipocyte areas were traced manually and analyzed with Win ROOF software (Mitani Co. Ltd., Chiba, Japan). White adipocyte areas were measured in 400 or more cells per mouse in each group according to methods previously described (8, 11).

RNA preparation, Northern blot analysis, RNase protection assay, phosphatidylinositol 3-kinase assay, immunoprecipitation, and immunoblotting. Total RNA was prepared from cells or tissues with TRIzol (Invitrogen Corp., Carlsbad, California, USA), according to the manufacturer's instructions. Total RNA from 5–10 mice in each group was pooled, and aliquots were subjected to Northern blot analysis with the probes for rat acyl-CoA oxidase (ACO) (a gift of T. Hashimoto), mouse CD36, uncoupling protein 2 (UCP2), PPARα, or resistin cDNA, or RNase protection assay was performed, using a standard protocol, to measure mRNAs of TNF-α (8, 11, 19). The radioactivity in each band was quantified, and the fold change in each mRNA was calculated after correction for loading differences by measuring the amount of 28S rRNA. Very low levels (< 10%) of aP2 mRNA were detected in muscle as compared with those in WAT (20). In contrast, at least comparable levels of CD36, SREBP1, SCD1, ACO, and UCP2 mRNAs were detected in muscle compared with those in WAT. These data suggest that the results of muscle tissue essentially represent those of muscle cells, despite the muscle being contaminated with a small amount (< 10%) of intermyocyte fat. The procedures used for phosphatidylinositol 3-kinase (PI 3-kinase) assay, immunoprecipitation, and immunoblotting were described previously (21). Representative data from one of three independent experiments are shown.

Cell culture, transfection, transactivation assays, and induction of adipocyte differentiation. CV-1 and 3T3L1 cells were cultured in DMEM with 10% FCS, and transfection and induction of adipogenic differentiation were carried out according to methods described previously (11, 22, 23). Heterodimers were formed by cotransfecting RXRα with an expression vector for PPARγ, PPARα, PPARδ, TR, RAR, or VDR. PPARγ/RXR, or PPARα/RXR, or PPARδ/RXR activity was assessed on a ACO PPRE-tk LUC (24), and RXR/TR, RXR/RAR, or RXR/VDR activity was tested respectively on DR-4, 5-tk LUC, and DR-3-tk-CAT, as described previously (22, 23).

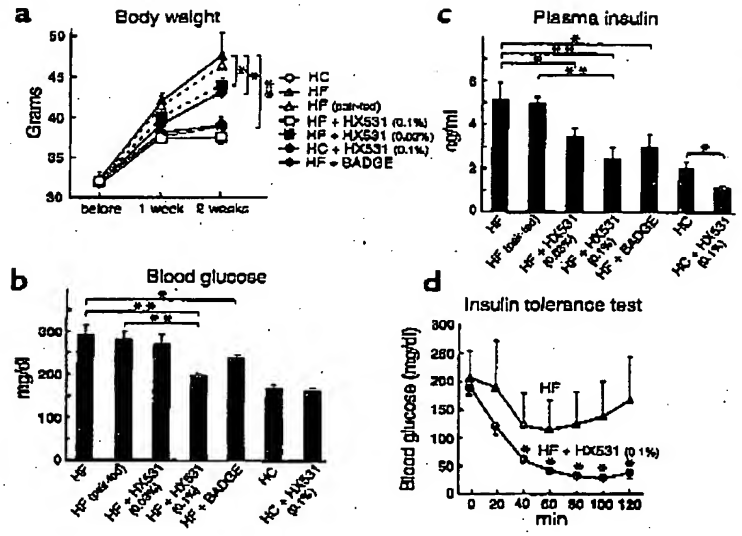

Figure 2
Both the RXR antagonist HX531 and the PPARγ antagonist BADGE exert antiobesity and antidiabetic effects in proportion to their potencies as PPARγ/RXR inhibitors in vitro (a–d). Body weight (a), fasting blood glucose (b), fasting plasma insulin (c), and insulin tolerance test (d) of KKAy mice untreated or treated with HX531 (+HX531) or BADGE (+BADGE) for 2 weeks while on the HF diet or the HC diet. HX531 or BADGE was given as an indicated percentage of food admixture. The same amounts of food were given to the pair-fed group as to mice treated with HX531, given as a 0.1% food admixture. Each bar represents the mean ± SE (n = 5–10). $^*P < 0.05$, $^{**}P < 0.01$ with versus without HX531 or BADGE.

Leptin treatment. Leptin treatment was performed as described previously (25). An Alzet micro-osmotic pump (model 1002, Alza Corp., Palo Alto, California, USA) was inserted subcutaneously into the back of each mouse. The pumps delivered 0 or 5 (low doses) or 50 μg (high dose) of mouse recombinant leptin (Sigma Chemical Co.) per day for 12 days in a total volume of 0.1 ml PBS.

Lipid metabolism. Liver and muscle homogenates were extracted, and their TG and fatty acyl-CoA (FA-CoA) content were determined as described previously (22, 26), with some modifications.

Results

HX531 serves as a PPARγ/RXR inhibitor. Because PPARγ functions as a heterodimer with RXR (1, 3, 5), we used a cotransfection reporter assay to screen compounds for responsiveness via the peroxisome proliferator response element (PPRE) and identified HX531 (17) as a PPARγ/RXR inhibitor (Figure 1a). In the presence of PPARγ agonists such as rosiglitazone (6) or 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2 (27, 28), RXR agonists such as LG100268 (7), or 9-*cis* retinoic acid (29, 30), or both, HX531 functioned as a partial inhibitor and produced a concentration-dependent decrease in transactivation by way of PPRE (24) (Figure 1a). We next analyzed the effects of HX531 on 3T3L1 adipocyte differentiation and found HX531 to be capable of inhibiting adipocyte differentiation in 3T3L1 cells induced by rosiglitazone, LG100268, or both, as well as by conventional hormonal stimuli (a combination of insulin, dexamethasone, and 3-isobutyl-1-methylxanthine) (Figure 1b).

By contrast, HX531 had no apparent effect on PPARα/RXR (1, 4) agonist-induced transactivation (Figure 1c) or on the expression of molecules induced by the PPARα agonist (18, 19, 22, 31, 32) Wy-14,643 in

Table 1
Effects of HX531 on other RXR partners in vitro and in vivo

			HX531		
			–	+	P
In vitro	PPARδ	transactivation (%)	100 ± 9	93 ± 7	NS
	RAR	transactivation (%)	100 ± 13	87 ± 19	NS
	TR	transactivation (%)	100 ± 9	96 ± 8	NS
	VDR	transactivation (%)	100 ± 11	106 ± 12	NS
In vivo	RAR	peripheral blood leukocytes (%)	58.3 ± 11.1	49.0 ± 4.2	NS
	TR	serum TSH levels (ng/ml)	3.85 ± 0.30	3.34 ± 0.93	NS
		serum fT3 levels (pg/ml)	2.74 ± 0.34	2.89 ± 0.32	NS
		serum fT4 levels (ng/dl)	1.87 ± 0.24	1.74 ± 0.21	NS

Transactivation analysis of PPARδ/RXR, RAR/RXR, TR/RXR, and VDR/RXR in vitro. CV-1 cells were treated with 100 nM of carbaprostacyclin, T3, all trans retinoic acid (atRA), or 1α, 25-dihydroxy-vitamin D3 (VD3) with or without 1 μM of HX531. The results are expressed as the percentage of the value of control cells without HX531. The percentages of peripheral blood leukocytes and serum TSH, free T3, and free T4 levels of KKAy mice untreated or treated with HX531 for 4 weeks while on the HF diet are shown. HX531 was given as a 0.1% food admixture. Values are mean ± SE (n = 5–10). NS, no significant difference, with versus without HX531.

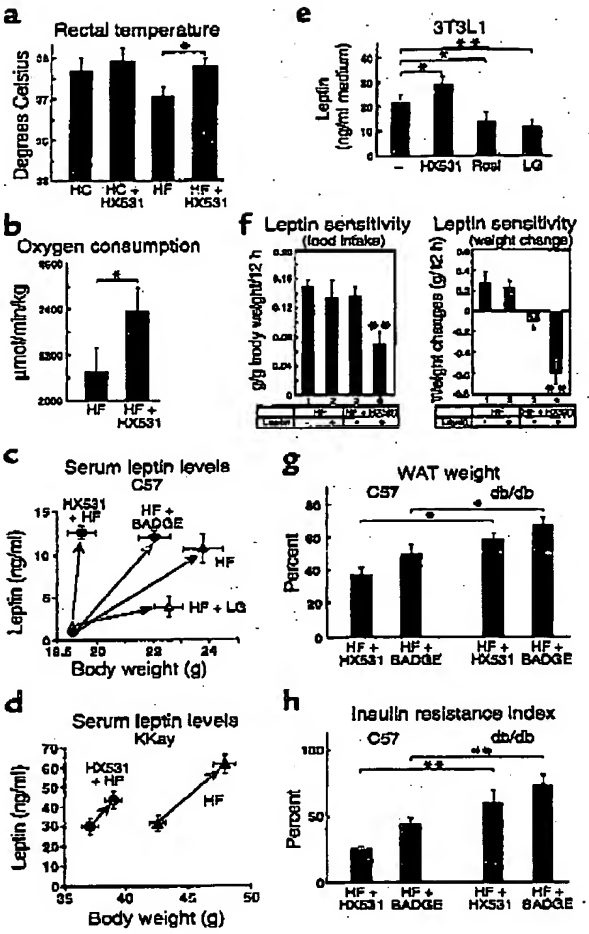

Figure 3
Both the RXR antagonist HX531 and the PPARγ antagonist BADGE exert antiobesity and antidiabetic effects in part through leptin-dependent pathways (a and b). Rectal temperature (a) and oxygen consumption (b) of KKAy mice untreated or treated with HX531 (+HX531) for 2 weeks while on the HF diet or the HC diet. Serum leptin levels of C57 (c) or KKAy mice (d) untreated or treated with HX531, BADGE (+BADGE), or LG100268 (+LG) for 2 weeks while on the HF diet. The left sides of the arrows are before-treatment (c) and after 1-week treatment (d) levels. (e) Leptin protein levels in the medium of 3T3L1 adipocytes treated with 10 μM HX531, 1 μM LG100268, or 100 nM rosiglitazone (Rosi) for 24 hours. (f) Effects of intraperitoneal leptin administration in untreated C57 mice or C57 mice treated with HX531 for 10 days while on the HF diet. Groups of untreated mice or mice treated with HX531 received an intraperitoneal injection of either leptin (10 μg/g/d) (+) or isotonic sodium chloride solution (–). Food intake/12 h (left) and weight changes/12 h (right) were measured. (g and h) WAT weight (g) and insulin resistance index (7) (h) of C57 and *db/db* mice untreated or treated with HX531 or BADGE for 2 weeks while on the HF diet. The results are expressed as the percentage of the value of untreated mice on the HF diet (g and h). Each bar represents the mean ± SE (n = 5–10). $^*P < 0.05$, $^{**}P < 0.01$, C57 versus *db/db* or untreated versus treated with HX531, BADGE, Rosi, LG, or leptin.

the hepatoma cell line Fao (Figure 1d and data not shown). It also had minimal effects on other RXR partners (33), e.g., PPARδ, thyroid hormone receptor (TR), retinoic acid receptor (RAR), and vitamin D receptor (VDR) in vitro and in vivo, including serum thyroid-stimulating hormone (TSH), free T3, and free T4 levels, the number of peripheral blood leukocytes, bone marrow blasts, osteoclasts, or osteoblasts, or the amount of osteoid in vivo (Table 1 and data not shown). These findings suggest that HX531 serves as a PPARγ/RXR inhibitor. As reported previously (16), BADGE acted as a relatively selective antagonist for PPARγ (Figure 1a), but did not inhibit PPARα (Figure 1c). However, a 100-fold higher dose of BADGE was required to show a similar potency of antagonistic activity in the transactivation assay specific to PPARγ/RXR (Figure 1a).

Both the RXR antagonist HX531 and the PPARγ antagonist BADGE exert antiobesity and antidiabetic effects in proportion to their potencies as PPARγ/RXR inhibitors in vitro. We investigated the effects of the RXR antagonist HX531 and the PPARγ antagonist BADGE on body weight, glucose, and insulin concentrations in KKAy mice (34) on both a HF and a high-carbohydrate (HC) diet. Untreated KKAy mice on the HF diet gained significantly more weight than the mice on the HC diet (Figure 2a). In contrast, treatment with HX531 or BADGE prevented a time-dependent increase in weight on the HF diet (Figure 2a). Treatment with the RXR antagonist or the PPARγ antagonist also prevented HF diet–induced hyperglycemia (Figure 2b) and hyperinsulinemia (Figure 2c). On the HF diet, the glucose-lowering effect of insulin was greater in mice treated with HX531 than in untreated mice (Figure 2d). These findings indicate that the RXR antagonist and the PPARγ antagonist have potential as antiobesity and antidiabetic drugs. Moreover, the potencies of HX531 and BADGE as antiobesity and antidiabetic drugs (Figure 2, a–c) appeared to be essentially proportional to their potencies as PPARγ/RXR inhibitors in vitro (Figure 1a).

Both the RXR antagonist HX531 and the PPARγ antagonist BADGE exert antiobesity and antidiabetic effects in part through leptin-dependent pathways. Food intake was slightly, though not significantly, lower in mice treated with the RXR antagonist HX531 and the PPARγ antagonist BADGE on the HF diet (Figure 3f, left, lanes 1 and 3 and data not shown). The observations that mice treated with HX531 were protected from HF diet–induced obesity (Figure 2a) and insulin resistance (Figure 2, b and c), even when compared with pair-fed controls, may indicate that HX531 increased energy expenditure. Indeed, rectal temperature was significantly higher (Figure 3a) and oxygen consumption was significantly increased in mice treated with HX531 (Figure 3b). We next investigated whether leptin (35) might account for these phenotypes in mice treated

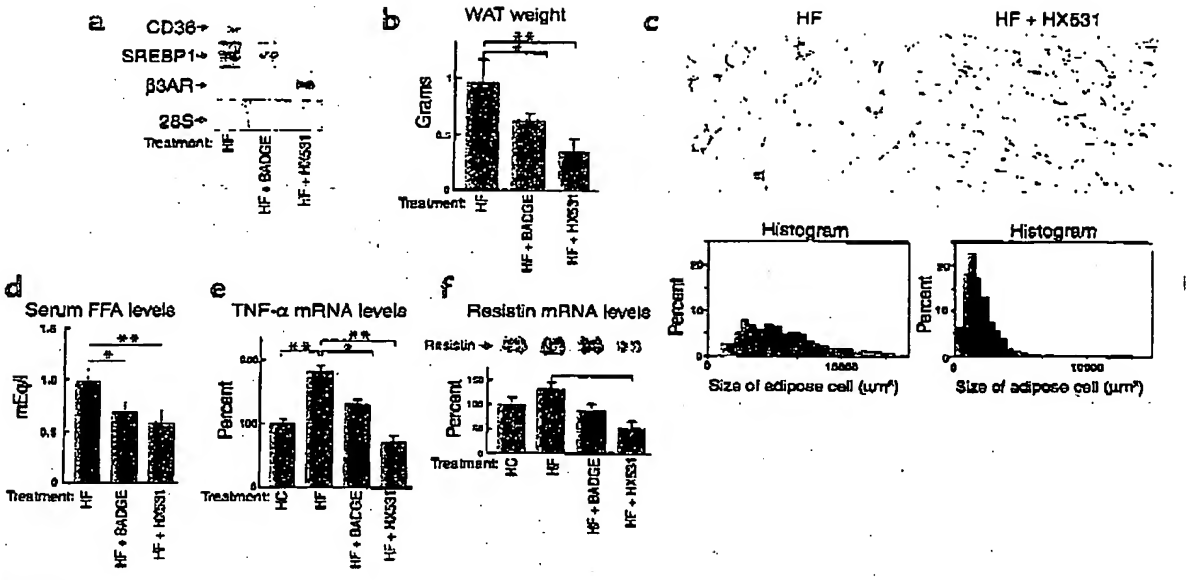

Figure 4

Both the RXR antagonist HX531 and the PPARγ antagonist BADGE reduce expressions of the molecules involved in fatty acid influx and lipogenesis and increase expression of β3-AR in WAT. (a) Amounts of the mRNAs of FAT/CD36, SREBP1, and β3-AR in WAT (a) of KKAy mice. (b and c) WAT mass (b), histological analysis (top), and cell size distribution (bottom) (c) of epididymal WAT from KKAy mice. (d–f) Serum FFA levels (d), TNF-α mRNA levels (e), and resistin mRNA levels in WAT (f) of KKAy mice, untreated (HF), treated with HX531 (HF + HX531), or with BADGE (HF + BADGE) for 2 weeks while on the HF diet. HX531 or BADGE was given as a 0.1% or 3% food admixture, respectively. Each bar represents the mean ± SE (n = 5–10). $^*P < 0.05$, $^{**}P < 0.01$, HF versus HF + HX531 or HF + BADGE.

with the PPARγ/RXR inhibitor. The serum leptin levels normalized by body weight in both normal wild-type (C57) and KKAy mice treated with the RXR antagonist HX531 or the PPARγ antagonist BADGE were higher than those of untreated mice (Figure 3, c and d). Expression and secretion of leptin into the medium by HX531-treated 3T3L1 adipocytes were also significantly higher than those by untreated cells in vitro (Figure 3e and data not shown), suggesting that the increase in serum leptin levels may be due to HX531-induced derepression of leptin gene transcription by PPARγ/RXR (36).

Moreover, leptin sensitivity as assessed by reductions in food intake and body weight in response to exogenously administered leptin was significantly increased in wild-type mice treated with HX531 (Figure 3f), even when compared with pair-fed controls (data not shown), raising the possibility that increased leptin effects may contribute to the effects of PPARγ/RXR inhibitors. In wild-type mice, HX531 or BADGE largely prevented HF diet–induced obesity and insulin resistance (Figure 3, g and h, left). In contrast, the antiobesity and antidiabetic effects of the RXR antagonist or the PPARγ antagonist were partially attenuated in *db/db* mice (35), which are leptin receptor deficient (Figure 3, g and h, right). These findings suggest that the RXR antagonist and the PPARγ antagonist exerted their antiobesity and antidiabetic effects through both leptin-dependent and independent pathways.

Both the RXR antagonist HX531 and the PPARγ antagonist BADGE decrease molecules involved in fatty acid influx into WAT by direct antagonism of PPARγ/RXR and concomitantly decrease lipogenic enzymes and increase β3-AR via leptin effects. In WAT, where PPARγ is expressed most predominantly, treatment with the RXR antagonist HX531 or the PPARγ antagonist BADGE reduced the expression of fatty acid translocase/CD36 (FAT/CD36) (ref. 37; Figure 4a), the promoter of which contains PPRE, indicating that HX531 and BADGE did indeed function as inhibitors of PPARγ/RXR in vivo. In WAT from mice treated with HX531 or BADGE, reduced expressions of lipogenic enzymes such as sterol regulatory element-binding protein 1 (SREBP1) (Figure 4a), stearoyl-CoA desaturase 1 (SCD1) (data not shown), and increased expression of β3-adrenergic receptor (β3-AR) (Figure 4a) were also observed in proportion to their increased leptin effects (Figure 3c and see Figure 7c) (38) and decreased PPARγ/RXR effects (Figure 1b) (39). These alterations of gene expression may in concert prevent adipocyte hypertrophy (Figure 4c) and reduce WAT mass (Figure 4b), and thereby obesity, on the HF diet. These data suggest that the RXR antagonist and the PPARγ antagonist prevented adipocyte hypertrophy, which ultimately alleviated insulin resistance via diminution of free fatty acids (FFA) (Figure 4d), TNF-α (Figure 4e) (40) and resistin (Figure 4f) (41), at least in part.

Both HX531 and BADGE decrease molecules involved in fatty acid influx into muscle by direct antagonism of PPARγ/RXR, and decrease lipogenic enzymes and increase fatty acid combustion and energy dissipation. In skeletal muscle in which PPARγ was relatively abundantly expressed, the RXR antagonist HX531 or the PPARγ antagonist BADGE also reduced expression of FAT/CD36 (Figure 5a). In addition, HX531 or BADGE decreased expressions of lipogenic enzymes such as SREBP1 (data not shown) and SCD1 (Figure 5a) and increased expressions of molecules involved in fatty acid combustion such as ACO, and energy dissipation, such as UCP2 (Figure 5a), in proportion to their increased leptin effects (Figure 3c and see Figure 7c) (38, 42) and decreased PPARγ/RXR effects (Figure 1b). These alterations of gene expression by HX531 or BADGE appeared to reduce long-chain FA-CoA (Figure 5b) and TG content in muscle (Figure 5c), thereby improving insulin signal transduction (43), as demonstrated by increases in insulin-induced tyrosine phosphorylation of the insulin receptor (IR), insulin receptor substrate 1 (IRS-1) and IRS-2, and insulin-stimulated PI 3-kinase activity in phosphotyrosine (PY), and IRS-1 and IRS-2 immunoprecipitates of skeletal muscle (Figure 5d).

Both the RXR antagonist HX531 and the PPARγ antagonist BADGE increase molecules involved in fatty acid combustion and energy dissipation via leptin and PPARα pathways in the liver and brown adipose tissue. In contrast, expression of FAT/CD36 was markedly increased in the livers of mice treated with the RXR antagonist HX531 or the PPARγ antagonist BADGE (Figure 6, a and b), where PPARα was predominantly expressed. Moreover, expression of enzymes involved in β-oxidation, such as peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (HD), ACO, and UCP2 were markedly increased in mice treated with HX531 or BADGE (Figure 6, a and b). These data strongly suggest PPARα pathways to be activated in the liver treated with the RXR antagonist or the PPARγ antagonist, since these effects were recapitulated by Wy-14,643, a PPARα agonist (Figure 6a) (18, 19, 22, 31). This HX531- or BADGE-induced activation of PPARα pathways presumably occurred via moderate functional antagonism of PPARγ/RXR, since PPARγ heterozygous deficiency also activated PPARα pathways (see Figure 8a, lane 4). Activation of PPARα pathways by HX531 or BADGE was partially abrogated in *db/db* mice (Figure 6a), suggesting that the RXR antagonist and the PPARγ antagonist exerted these effects, in part, through leptin signaling pathways (1, 42). Moreover, expressions of lipogenic enzymes such as SREBP1 (Figure 6b) and SCD1 (data not shown) were markedly reduced, possibly as a consequence of increased leptin levels (Figure 3c and Figure 7c) (38, 42).

Marked elevations of enzymes involved in β-oxidation and UCP2, as well as this reduction of lipogenic enzymes (Figure 6, a and b), appeared to significantly reduce liver weight (data not shown) and FA-CoA content (Figure 6c) and to ameliorate severe fatty liver (Figure 6d) (25, 34) in wild-type mice treated with HX531 or BADGE while on the HF diet. The reduction of FA-CoA and amelioration of fatty liver by HX531 were associated with increased expression of glucokinase (GK), decreased expression of enzymes involved in gluconeogenesis, such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-

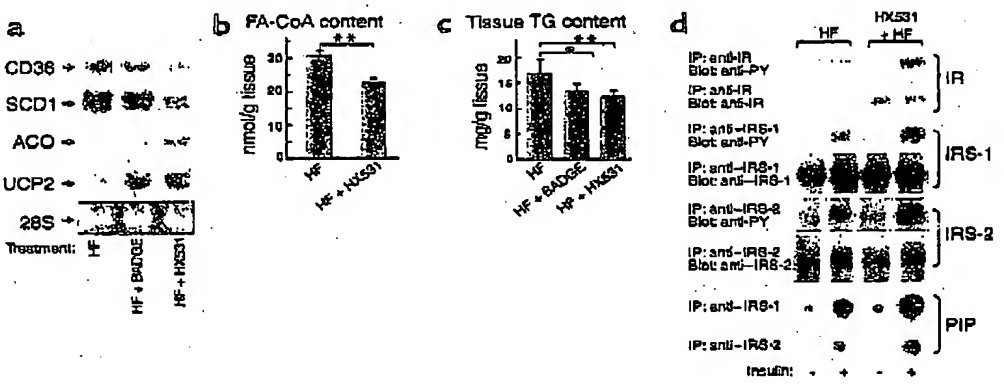

Figure 5

The RXR antagonist HX531 and the PPARγ antagonist BADGE both decrease molecules involved in fatty acid influx and lipogenesis and increase molecules involved in energy consumption in skeletal muscle. (a) Amounts of FAT/CD36, SCD1, ACO, and UCP2 in skeletal muscles (a) of KKAy mice. (b–d) FA-CoA (b) and TG (c) content and expression and insulin-induced tyrosine phosphorylation of IR, IRS-1 and -2, and insulin-stimulated PI 3-kinase activity (d) in skeletal muscles of KKAy mice, untreated (HF) or treated with HX531 (HF + HX531) or with BADGE (HF + BADGE) for 2 weeks while on the HF diet. (b and c) Tissue homogenates were extracted, and their TG and FA-CoA content was determined as described previously (22, 26), with some modifications. (d) Mice were stimulated with or without 1 Ug^{-1} body weight of insulin for 2 minutes. Lysates were immunoprecipitated (IP) with the Ab's indicated, followed by immunoblotting with the Ab's indicated or kinase assay for PI. Labeled PI (PIP) was subjected to thin-layer chromatography and autoradiography as described previously (21). HX531 or BADGE was given as a 0.1% or 3% food admixture, respectively. Each bar represents the mean ± SE (n = 5–10). *P < 0.05, **P < 0.01, HF versus HF + HX531 or HF + BADGE.

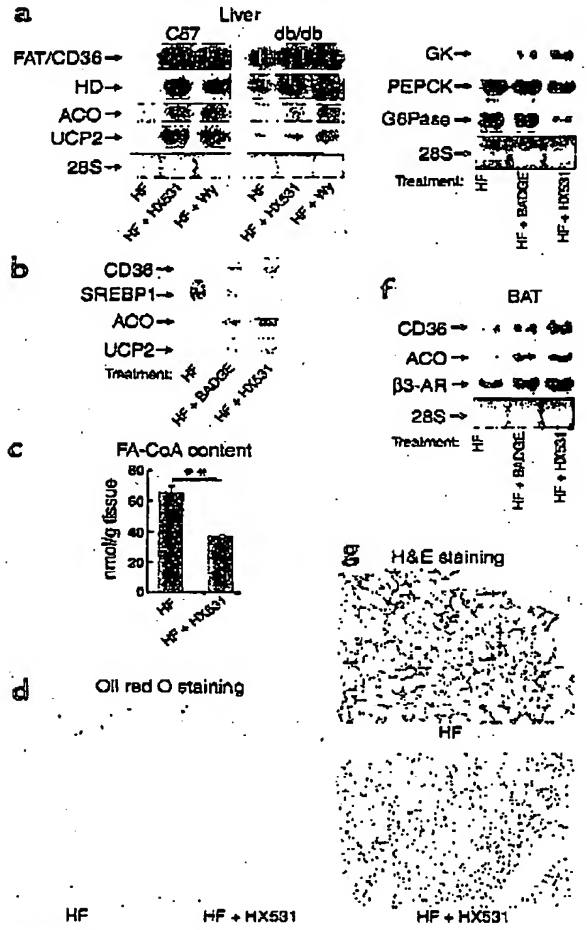

Figure 6

The RXR antagonist HX531 and the PPARγ antagonist BADGE both increase molecules involved in fatty acid combustion and energy dissipation via leptin and PPARα pathways in the liver and BAT (a, b, e, f). Amounts of the mRNAs of FAT/CD36, SREBP1, HD (peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase), ACO, UCP2, and β3-AR in livers from C57 and *db/db* mice (a), in livers from KKAy mice (b), in BAT from KKAy mice (f), and glucokinase, PEPCK, and G6Pase in livers from KKAy mice (e). (c, d, g) Hepatic FA-CoA content (c), hepatic TG content (oil red O staining) (d), size of brown adipocytes from KKAy mice (g), untreated (HF) or treated with HX531 (HF + HX531), Wy-14,643 (HF + Wy), or BADGE (HF + BADGE) for 2 weeks while on the HF diet. HX531, or Wy-14,643, or BADGE was given as a 0.1%, 0.01%, or 3% food admixture, respectively. Each bar represents the mean ± SE (n = 5–10). $^*P < 0.05$, $^{**}P < 0.01$, HF versus HF + HX531 or HF + BADGE.

phosphatase (G6Pase) (Figure 6e), and increased glycogen content (34) (data not shown), indicating increased insulin actions in the liver (43).

In brown adipose tissue (BAT), where PPARα was relatively abundantly expressed as compared with WAT, expression of FAT/CD36 was increased by treatment with the RXR antagonist HX531 (Figure 6f) in contrast to WAT (Figure 4a). HX531 or BADGE also increased expression of ACO, which is primarily involved in the regulation of β-oxidation in BAT, and β3-AR (Figure 6f). The increased expressions of CD36 and ACO by the RXR antagonist or the PPARγ antagonist appeared to be caused by activation of PPARα pathways (19, 22, 42), similar to those in the liver. On the other hand, the increased expression of β3-AR appeared to be a direct effect of increased leptin signaling (Figure 3c and Figure 7c) (38) and decreased PPARγ/RXR effects (Figure 1b and Figure 4a) (39). Increases in the expression of these molecules in concert may provide the mechanism whereby BAT mass and brown adipocyte hypertrophy were reduced (Figure 6g) in mice treated with HX531 or BADGE while on the HF diet. This is consistent with increased energy expenditure and protection against adipocyte hypertrophy by RXR antagonist or PPARγ antagonist treatment.

Treatment of heterozygous PPARγ-deficient mice with an RXR antagonist or a PPARγ antagonist results in re-emergence of hyperglycemia and insulin resistance associated with lipoatrophy. To investigate the effects of the RXR antagonist or the PPARγ antagonist on mice with moderately reduced PPARγ activity, we studied the phenotypes of HX531- or BADGE-treated heterozygous PPARγ-deficient mice as compared with those of untreated wild-type, HX531-, or BADGE-treated wild-type mice on the HF diet. Moderately reduced expression of CD36 in WAT was observed in wild-type mice treated with BADGE or HX531 (~40 or 70%, respectively) (Figure 7a, lanes 2 and 3), suggesting the possibility that PPARγ/RXR activity may be moderately decreased in wild-type mice treated with BADGE or HX531. Treatment of heterozygous PPARγ-deficient mice with BADGE or HX531 resulted in further decreased expression of CD36 in WAT (Figure 7a, lanes 4 and 5), raising the possibility that PPARγ/RXR activity may be severely decreased in heterozygous PPARγ-deficient mice treated with BADGE or HX531. Wild-type mice treated with BADGE or HX531 were protected from HF diet–induced increases in WAT mass (Figure 7b, lanes 2 and 3). Administration of BADGE or HX531 to heterozygous PPARγ-deficient mice for 4 weeks resulted in disappearance of visible WAT, i.e., lipoatrophy (Figure 7b, lanes 5 and 6) (25, 44, 45).

Wild-type mice treated with BADGE or HX531 showed higher serum leptin levels than untreated wild-type mice on the HF diet (Figure 7c, lanes 1–3), presumably due to decreased PPARγ/RXR-mediated suppression of leptin gene transcription (36). The serum leptin levels of heterozygous PPARγ-deficient mice treated with BADGE or HX531 were, however, markedly reduced (Figure 7c, lanes 5 and 6) as a result of WAT depletion (Figure 7b, lanes 5 and 6) (19, 44).

Wild-type mice treated with BADGE or HX531 were protected from HF diet–induced hyperglycemia and insulin resistance (Figure 7d, lanes 2 and 3). Paradoxically, treatment of heterozygous PPARγ-deficient mice

with BADGE or HX531 resulted in a re-emergence of hyperglycemia and insulin resistance (Figure 7d, lanes 5 and 6) (25, 44, 45, 46), as compared with wild-type mice treated with BADGE or HX531 on the HF diet.

The combination of leptin deficiency and decreased effects of PPARα pathways leads to insulin resistance in heterozygous PPARγ-deficient mice without WAT induced by treatment with RXR antagonist or a PPARγ antagonist. BADGE- or HX531-treated wild-type mice exhibited decreased hepatic expression of lipogenic enzymes such as SREBP1 (data not shown) and SCD1 (Figure 8a, lanes 2 and 3) by increased serum leptin levels (Figure 7c, lanes 2 and 3). Moreover, these mice showed increased expression of UCP2 and enzymes involved in β-oxidation (Figure 8a, lanes 2 and 3), presumably by increased activation of PPARα pathways (Figure 6a). In marked contrast, induction of SREBP1 (data not shown) and SCD1 (Figure 8a, lanes 5 and 6) due to markedly decreased serum leptin levels (Figures 7c, lanes 5 and 6) (38, 42) and decreased effects of PPARα pathways, such as reduced expressions of ACO and UCP2 (Figure 8a, lanes 4 and 5, and data not shown) (18, 19, 22, 31), were observed in heterozygous PPARγ-deficient mice treated with BADGE or HX531. These alterations of gene expression in concert appeared to increase tissue TG content in heterozygous PPARγ-deficient mice treated with BADGE or HX531 (Figure 8b, lanes 5 and 6), in contrast to the decreased tissue TG content seen in wild-type mice treated with BADGE or HX531 (Figure 8b, lanes 2 and 3).

Similarly, in skeletal muscle, treatment of heterozygous PPARγ-deficient mice with BADGE or HX531 increased expressions of SREBP1 (Figure 8c, lanes 5 and 6) and SCD1 (data not shown), decreased expressions of ACO and UCP2 (Figure 8c, lanes 5 and 6) and concomitantly increased the tissue TG content (Figure 8d, lanes 5 and 6) as compared with treatment of wild-type mice with BADGE or HX531 (Figure 8, c and d, lanes 2 and 3).

To determine the relative contributions of leptin deficiency and decreased effects of PPARα pathways to the insulin resistance observed in heterozygous PPARγ-deficient mice without WAT induced by treatment with HX531or BADGE, we supplied the mice with leptin and/or Wy-14,643, a PPARα agonist. Very low plasma leptin levels were restored to almost the control value by continuous systemic infusion of a low dose of recombinant leptin (Figure 8e, left, lanes 2 and 3), which partially alleviated insulin resistance (Figure 8e, right, lanes 2 and 3). We found that Wy-14,643 treatment also partially reversed the insulin resistance seen in HX531- or BADGE-treated heterozygous PPARγ-deficient mice (Figure 8e, right, lanes 2 and 5). Interestingly, combining physiological doses of leptin and Wy-14,643 almost completely ameliorated insulin resistance (Figure 8e, right, lanes 2 and 6), presumably due to reduced tissue TG content (data not shown). These data suggested that leptin deficiency and decreased effects of PPARα pathways caused insulin resistance by different mechanisms, at least to some extent. However, the insulin resistance in these mice could be completely overcome by continuous infusion of a high dose of leptin (Figure 8e, right, lanes 2 and 4). Interestingly, we very recently found that in combination with leptin, physiological dose of adiponectin ameliorated insulin resistance observed in our lipotrophic mice presumably through activation of PPARγ pathways (47).

Discussion

Effect of the RXR antagonist HX531 and the PPARγ antagonist BADGE on the regulation of insulin sensitivity. This study demonstrated that treatment of wild-type mice with the RXR antagonist HX531 or the PPARγ antago-

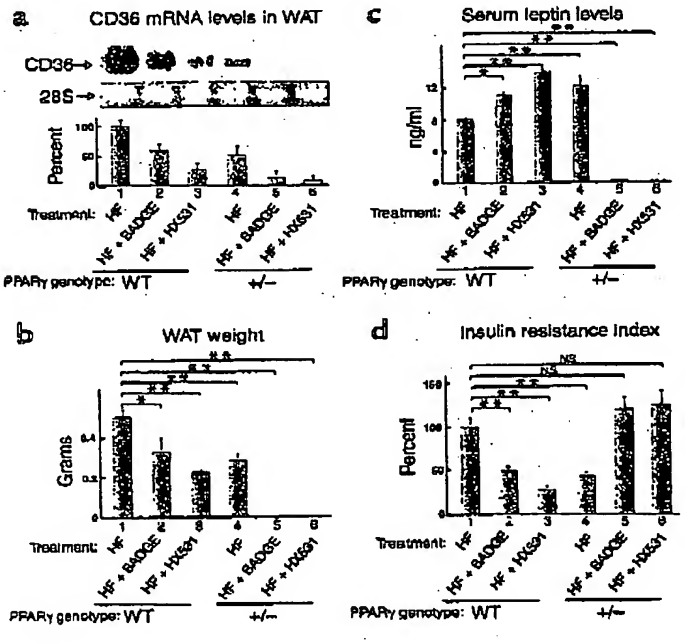

Figure 7
Treatment of heterozygous PPARγ-deficient mice with an RXR antagonist or PPARγ antagonist results in re-emergence of hyperglycemia and insulin resistance associated with lipoatrophy. (a) Amounts of the mRNAs of FAT/CD36 in WAT. (b–d) WAT mass (b), serum leptin levels (c), insulin resistance index (d), of wild-type (WT) and heterozygous PPARγ-deficient mice (+/−) untreated (−) or treated with HX531 or BADGE for 3 weeks (a) or for 4 weeks (b–d) while on the HF diet. HX531 or BADGE was given as a 0.1% or 3% food admixture, respectively. Each bar represents the mean ± SE (n = 5–10). $^*P < 0.05$, $^{**}P < 0.01$, untreated versus treated with HX531 or BADGE or compared with untreated wild-type mice. NS, no significant difference.

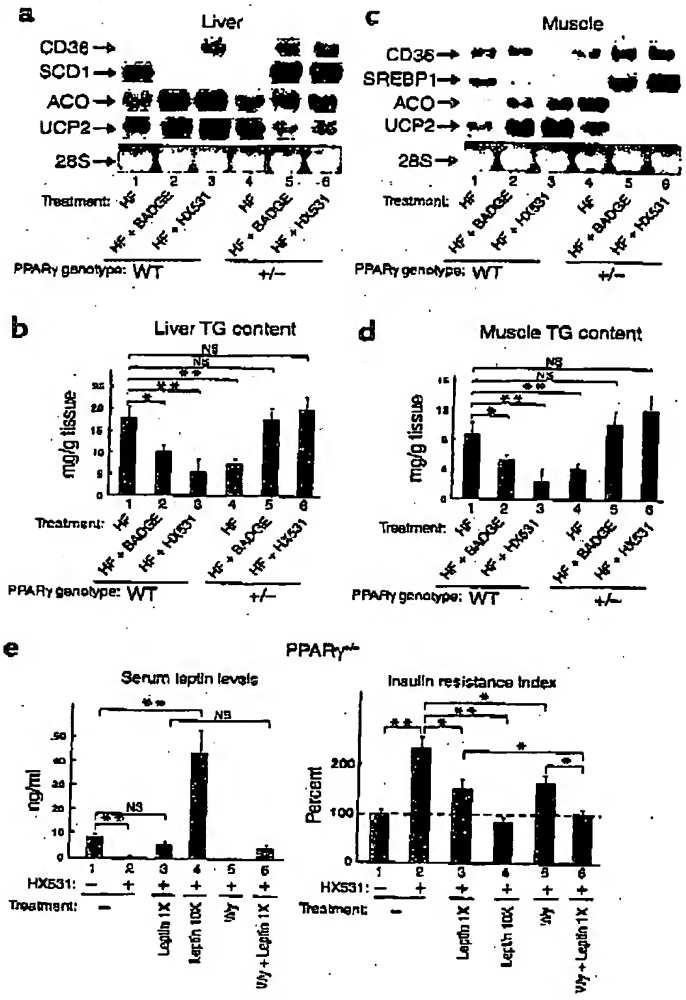

Figure 8

The combination of leptin deficiency and decreased effects of PPARα pathways results in insulin resistance in heterozygous PPARγ-deficient mice without WAT induced by treatment with an RXR antagonist or a PPARγ antagonist (a and c). Amounts of the mRNAs of FAT/CD36, SREBP1, SCD1, ACO, and UCP2 in the liver (a) and in skeletal muscle (c). (b and d) TG content of the livers (b) and skeletal muscles (d) of wild-type (WT) and heterozygous PPARγ-deficient mice (+/−) untreated (−) or treated with HX531 or BADGE for 4 weeks (a–d) while on the HF diet. (e) Serum leptin levels (left) and insulin resistance index (right) of heterozygous PPARγ-deficient mice (PPARγ$^{+/-}$) untreated (−) or treated with HX531 (+) for 6 weeks or PPARγ$^{+/-}$ treated with low doses of leptin (Lep1X), high doses of leptin (Lep10X), Wy-14,643 (Wy), or a combination for the last 12 days of a 6-week HX531 treatment while on the HF diet. HX531 or Wy-14,643 was given as a 0.1 or 0.01% food admixture, respectively. The results are expressed as the percentage of the value of untreated PPARγ$^{+/-}$ on the HF diet (right). HX531 or Wy-14,643 was given as a 0.1 or 0.01% food admixture, respectively. Each bar represents the mean ± SE (n = 5–10). $^*P < 0.05$, $^{**}P < 0.01$, untreated versus treated with HX531, Lep1X, Lep10X, or Wy, or compared with untreated wild-type mice. NS, no significant difference.

nist BADGE prevented HF diet-induced obesity, insulin resistance, and diabetes by reducing tissue TG content in WAT, skeletal muscle, and the liver by at least three mechanisms. First, the RXR antagonist HX531 and the PPARγ antagonist BADGE reduced expression of PPARγ target genes such as CD36 in tissues where PPARγ was expressed predominantly (Figure 4a and 5a), which could limit fatty acid influx into WAT and skeletal muscle (19, 37). Second, the RXR antagonist and the PPARγ antagonist increased serum leptin levels and leptin sensitivity (Figure 3, c, d, f and 7c) and indeed increased its effects, i.e., reduced lipogenic enzymes and induction of β3-AR (Figures 4a, 5a, 6b and 6f) (38), as genetically demonstrated by the significant attenuation of their antiobesity and antidiabetic effects in leptin receptor–deficient mice (Figure 3, g and h). In KKAy mice, however, it is difficult to judge whether increased leptin effects could play a predominant role in driving the decreased weight gain and metabolic effects resulting from HX531 treatment, raising the alternative possibility that in KKAy mice leptin-independent pathways may play more role in these effects. Third, HX531 and BADGE increased expression of PPARα target genes such as ACO and UCP2 (Figures 5a and Figure 6, a, b, and f) in tissues in which PPARα was expressed, thereby increasing fatty acid combustion and energy dissipation in the liver, BAT, and skeletal muscle; these observations fit well with recently demonstrated effects of PPARα agonists on insulin resistance (48) and decreased fatty acid combustion in PPARα-deficient mice (49).

All three effects, downregulation of molecules involved in TG accumulation and activation of leptin and PPARα pathways, appear probably to be consequences of moderate reduction of PPARγ/RXR activity for the following two reasons. First, the RXR antagonist HX531 and the PPARγ antagonist BADGE exerted these three effects essentially in proportion to their potencies as PPARγ/RXR inhibitors in vitro. Second, with respect to these three effects, untreated heterozygous PPARγ-deficient mice exhibited the same molecular pathophysiology as wild-type mice treated with HX531 or BADGE (Figures 7 and 8). Very recently, mice lacking RXRα in adipocytes have also been reported to be resistant to HF diet–induced obesity (50). Although the authors speculated that RXRα mediated this effect as a heterodimeric partner for

PPARγ, the possibility could not be excluded that HX531 may mediate some of the effects that were observed in this study, particularly in vivo, by acting on other heterodimeric partners for RXR. Previously, HX531 was reported to be an antagonist of RXR homodimers and of the RAR/RXR heterodimer on the basis of inhibitory activity on transactivation assay in COS-1 cells (17). Although it had minimal effects on the numbers of peripheral blood leukocytes and bone marrow blasts in vivo, interaction with these receptor dimers may account for the present findings to some extent. Thus, the relative contributions of PPARγ, RXR, and/or their cognate endogenous ligands, as well as the potential contributions of other heterodimeric partners for RXR in mediating all the effects of HX531 or BADGE, will require further study.

On the basis of our data, we would like to propose the following possible hypothesis that may explain the mechanisms by which PPARγ/RXR regulates insulin sensitivity. On the HF diet, "normal" amounts of PPARγ/RXR activity seen in wild-type mice increases TG content in WAT, skeletal muscle, and the liver due to a combination of increased fatty acid influx into these tissues and HF diet–induced leptin resistance, leading to insulin resistance associated with obesity. By contrast, the moderate reduction of PPARγ/RXR activity observed in HX531- or BADGE-treated wild-type mice decreases the TG content in WAT, skeletal muscle, and the liver due to a combination of direct antagonism of PPARγ/RXR to limit fatty acid influx into WAT and skeletal muscle and increased leptin expression by antagonism of PPARγ/RXR-mediated suppression of the gene, thereby reducing expression of lipogenic enzymes. Moreover, consequent activation of the PPARα pathway in the liver, BAT, and skeletal muscle, increases expression of UCP2 and enzymes involved in β-oxidation. These alterations lead to prevention of HF diet–induced obesity and insulin resistance. Severely reducing PPARγ/RXR activity, however, depletes WAT in part due to severely decreased flux of FFA into WAT by marked suppression of target genes of PPARγ/RXR, leading to increased FA influx into liver/muscle and simultaneously to both leptin deficiency and decreased effects of PPARα pathways. These alterations lead to increased expression of lipogenic enzymes and decreased expression of enzymes involved in β-oxidation and UCP2, thereby markedly increasing skeletal muscle and liver tissue TG content, which can account for the re-emergence of insulin resistance in the face of lipoatrophy. The results obtained in this study may collectively raise the possibility that PPARγ/RXR regulates fuel partitioning among tissues, the net effects of leptin and PPARα activity, thereby playing an important role in the regulation of insulin sensitivity.

The appropriate level of PPARγ/RXR activity for insulin sensitivity. There may be an appropriate level of PPARγ/RXR activity for insulin sensitivity. Taken together, our data raise the possibility that there may be a hitherto unrecognized U-shaped relationship between PPARγ/RXR activity and insulin resistance within physiologically "normal" limits (Figure 9). The relationship between PPARγ/RXR activity and WAT mass may be linear. There may be an appropriate level of PPARγ/RXR activity for insulin sensitivity, which may be approximately 30–60% of "normal." Increases in PPARγ/RXR activity are associated with decreased serum leptin levels due to increased PPARγ/RXR-mediated suppression of leptin gene transcription, and decreases in PPARγ/RXR activity may be associated with decreased serum leptin levels due to WAT depletion. Thus, the relationship between PPARγ/RXR activity and leptin appears to exhibit an inverted U-shaped curve. Thus, impairment of the leptin pathway may be closely paralleled by impairment of insulin sensitivity.

HF diet–induced obesity is the major risk factor for diabetes and cardiovascular diseases, the prevalences of which are increasing dramatically. Although genetic evidence has strongly suggested PPARγ to act as a thrifty gene (5, 11), the molecular mechanisms accounting for this function of the gene were unclear (2, 51). We have shown herein that PPARγ/RXR promotes fat storage in the body by a combination of direct induction of molecules involved in TG accumulation and suppression of leptin gene expression as well as inactivation of PPARα–signaling pathways. In times of fasting, this PPARγ/leptin/PPARα network maximizes energy storage, which is quite advantageous for survival. In times of feast, which are the norm in industrialized nations nowadays, however, this network caus-

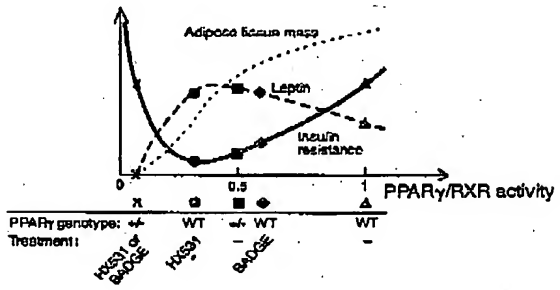

Figure 9
One possible model for the relationship between PPARγ/RXR activity and insulin sensitivity on the HF diet. The relationship between PPARγ/RXR activity and WAT mass may be linear. There may appear to be an optimal level of PPARγ/RXR activity for insulin sensitivity that is approximately 0.3–0.5 times normal. Increases in PPARγ/RXR activity as compared with the optimal range are associated with decreased serum leptin levels due to increased PPARγ/RXR-mediated suppression of leptin gene transcription, and decreases in PPARγ/RXR activity may be associated with decreased serum leptin levels due to depletion of WAT. Thus, these data raise the possibility that the relationship between PPARγ/RXR activity and leptin is an inverted U-shaped curve. The relationship between PPARγ/RXR activity and insulin resistance appears to exhibit such a U-shaped curve. Thus, impairment of the leptin pathway may closely parallel impairment of insulin sensitivity.

es excessive adiposity, insulin resistance, and obesity-related diseases such as diabetes. Thus appropriate antagonism of PPARγ/RXR, which simultaneously leads to appropriate agonism of leptin and PPARα, may be a logical approach to protection against obesity and related diseases such as type 2 diabetes. In this respect, treating subjects with the wild-type PPARγ (Pro 12 allele) with higher activity with PPARγ/RXR inhibitors is an example for "personalized treatment" of subjects genetically susceptible to obesity and diabetes. In contrast, our data raise the possibility that treating subjects with the variant PPARγ (Ala 12 allele) with lower PPARγ activity with PPARγ/RXR inhibitors may worsen their insulin resistance (46).

Although not shown in this study, we have also found that supraphysiological activation of PPARγ, far beyond 1 (PPARγ activity in wild-type mice on the HF diet), by TZD induces adipocyte differentiation, thereby increasing the number of small adipocytes (8). This in turn promotes a flux of FFA from the liver and muscle into WAT, leading to decreased liver and muscle TG content and improvement of insulin sensitivity at the expense of increased WAT mass, i.e., obesity (52). However, PPARγ activators including TZD have been reported to prevent atherosclerosis by reducing lipid accumulation in foamy arterial macrophages and also to inhibit cytokine production and inflammation, raising the possibility that PPARγ inhibitors promote atherosclerosis (53–55). Thus, like tissue-specific agonist/antagonist for the estrogen receptor tamoxifen, a tissue-specific PPARγ agonist/antagonist that functions as an agonist in arterial macrophages and simultaneously antagonizes PPARγ in adipose tissue may be the ultimate drug for treating obesity, type 2 diabetes, and atherosclerosis.

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The Mechanisms by Which Both Heterozygous Peroxisome Proliferator-activated Receptor γ (PPARγ) Deficiency and PPARγ Agonist Improve Insulin Resistance*

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Peroxisome proliferator-activated receptor (PPAR) γ is a ligand-activated transcription factor and a member of the nuclear hormone receptor superfamily that is thought to be the master regulator of fat storage; however, the relationship between PPARγ and insulin sensitivity is highly controversial. We show here that supraphysiological activation of PPARγ by PPARγ agonist thiazolidinediones (TZD) markedly increases triglyceride (TG) content of white adipose tissue (WAT), thereby decreasing TG content of liver and muscle, leading to amelioration of insulin resistance at the expense of obesity. Moderate reduction of PPARγ activity by heterozygous PPARγ deficiency decreases TG content of WAT, skeletal muscle, and liver due to increased leptin expression and increase in fatty acid combustion and decrease in lipogenesis, thereby ameliorating high fat diet-induced obesity and insulin resistance. Moreover, although heterozygous PPARγ deficiency and TZD have opposite effects on total WAT mass, heterozygous PPARγ deficiency decreases lipogenesis in WAT, whereas TZD stimulate adipocyte differentiation and apoptosis, thereby both preventing adipocyte hypertrophy, which is associated with alleviation of insulin resistance presumably due to decreases in free fatty acids, and tumor necrosis factor α, and up-regulation of adiponectin, at least in part. We conclude that, although by different mechanisms, both heterozygous PPARγ deficiency and PPARγ agonist improve insulin resistance, which is associated with decreased TG content of muscle/liver and prevention of adipocyte hypertrophy.

Peroxisome proliferator-activated receptor (PPAR)[1] γ is a ligand-activated transcription factor and a member of the nuclear hormone receptor superfamily that functions as a heterodimer with a retinoid X receptor (RXR) (1–5). Agonist-induced activation of PPARγ/RXR is known to increase insulin sensitivity (6, 7). Thiazolidinediones (TZD), which have the ability to directly bind and activate PPARγ (6) and to stimulate adipocyte differentiation (2, 8), are used clinically to reduce insulin resistance and hyperglycemia in type 2 diabetes (1, 2, 4, 5). We and others (9, 10) have reported that heterozygous PPARγ-deficient mice are protected from high fat (HF) diet- or aging-induced adipocyte hypertrophy, obesity, and insulin resistance. Consistent with this, the Pro-12 → Ala polymorphism in human PPARγ2, which moderately reduces the transcriptional activity of PPARγ, has been shown to confer resistance to type 2 diabetes (11–18). This apparent paradox raises the following important unresolved issue (14) which we addressed experimentally in this study. We attempted to explain how insulin resistance could be improved by two opposite PPARγ activity states, supraphysiological activation of PPARγ and moderate reduction. We did so by using heterozygous PPARγ-deficient mice and a pharmacological activator of PPARγ in wild-type mice.

We show here that supraphysiological activation of PPARγ by TZD markedly increases triglyceride (TG) content of white adipose tissue (WAT), thereby decreasing TG content of liver and muscle, leading to amelioration of insulin resistance at the expense of obesity. Moderate reduction of PPARγ activity by heterozygous PPARγ deficiency decreases TG content of WAT, skeletal muscle, and liver due to increased leptin expression and increase in fatty acid combustion and decrease in lipogenesis, thereby ameliorating HF diet-induced obesity and insulin resistance. Moreover, although heterozygous PPARγ deficiency and TZD have opposite effects on total WAT mass, heterozygous PPARγ deficiency decreases lipogenesis in WAT, whereas TZD stimulate adipocyte differentiation and apoptosis, thereby both preventing adipocyte hypertrophy. This results in a decrease in molecules causing insulin resistance such as free fatty acids (FFA) (15) and tumor necrosis factor (TNF) α (16) and

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[1] The abbreviations used are: PPARγ, peroxisome proliferator-activated receptor; WAT, white adipose tissue; TG, triglyceride; TZD, thiazolidinediones; RXR, retinoid X receptor; FFA, free fatty acids; TNF, tumor necrosis factor; HC, high carbohydrate; SREBP, sterol regulatory element-binding protein; IRS, insulin receptor substrate; HF, high fat; PI, phosphatidylinositol; BAT, brown adipose tissue.

up-regulation of insulin-sensitizing hormone adiponectin (17), thereby leading to alleviation of insulin resistance. We conclude that, although by different mechanisms, both heterozygous PPARγ deficiency and PPARγ agonist improve insulin resistance, which is associated with decreased TG content of muscle/liver and prevention of adipocyte hypertrophy.

EXPERIMENTAL PROCEDURES

Chemicals—Rosiglitazone was synthesized as described elsewhere (8). Wy-14,643 was purchased from Biomol (Plymouth Meeting, PA). All other materials were from the sources given in Refs. 8 and 9.

Animals, in Vivo Glucose Homeostasis, Assay of Endogenous Serum Leptin Concentrations, and Leptin Sensitivity—Heterozygous PPARγ-deficient mice have been described (9). All other animals were purchased from Nippon CREA Co., Ltd. Six-week-old mice were fed powdered chow according to methods described previously (9). Drugs were given as food admixtures (8, 9), and there was no toxicity observed including liver damage. The area of glucose and insulin curves was calculated by multiplying the cumulative mean height of the glucose values (1 mg ml^{-1} = 1 cm) and insulin values (1 ng ml^{-1} = 1 cm), respectively, by time (60 min = 1 cm) as described in Ref. 7. The results are expressed as the percentage of the value of each controls. The insulin resistance index (7) was calculated from the product of the areas of glucose and insulin × 10^{-2} in glucose tolerance test (9). The results are expressed as the ratio of the value of each wild-type controls on the high carbohydrate (HC) diet (9). Leptin was assayed with the enzyme-linked immunosorbent assay-based Quantikine M mouse leptin immunoassay kit (R & D Systems) according to the manufacturer's instructions. For leptin sensitivity (9), leptin (PeproTech) was administered to mice as a daily intraperitoneal injection of 10 μg/g body weight/day. Isotonic sodium chloride solution was administered to the controls. Food intake and body weight were measured to assess the effects of leptin administration.

Histological Analysis of Adipose Tissue and Determination of Adipocyte Size—Adipose tissue was removed from each animal, fixed in 10% formaldehyde/phosphate-buffered saline, and maintained at 4 °C until used. Fixed specimens were dehydrated, embedded in tissue-freezing medium (Tissue-Tek OCT compound; Miles), and frozen in dry ice and acetone. WAT was cut into 10-μm sections, and the sections were mounted on silanized slides. The adipose tissue was stained with hematoxylin and eosin. Mature white adipocytes were identified by their characteristic multilocular appearance. Total adipocyte areas were traced manually and analyzed with Win ROOF software (Mitani Co., Ltd., Chiba, Japan). White adipocyte areas were measured in 400 or more cells per mouse in each group according to the methods described previously (8, 9). Sections of adipose tissues from mice treated for 14 days were stained by the terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling technique with a kit (*In Situ* Cell Death Detection Kit, AP; Roche Molecular Biochemicals) to detect apoptotic nuclei as described (8), with slight modifications. The numbers of all nuclei and apoptosis positive-stained nuclei were counted to calculate the ratio to the number of apoptotic nuclei to total number of nuclei.

RNA Preparation, Northern Blot Analysis, RNase Protection Assay, PI3-Kinase Assay, Immunoprecipitation, and Immunoblotting—Total RNA was prepared from tissues with TRIzol (Life Technologies, Inc.) according to the manufacturer's instructions. Total RNA from 5 to 10 mice in each group was pooled, and aliquots were subjected to Northern blot analysis with the probes for rat acyl-CoA oxidase (Dr. T. Hashimoto), mouse CD36, UCP2, and adiponectin cDNA or RNase protection assay to measure mRNAs of TNFα performed using a standard protocol (8, 9, 18, 19). The radioactivity in each band was quantified, and the fold change in each mRNA was calculated after correction for loading differences by measuring the amount of 28 S rRNA. Very low levels (<10%) of adipocyte P2 mRNA were detected in muscle as compared with those in WAT. By contrast, CD36, SCD1, acyl-CoA oxidase, and UCP2 mRNAs were detected in muscle at levels comparable to those in WAT. These findings suggest that the results for muscle tissue essentially represent the results for the muscle cells, although the muscle was contaminated by a small amount (<10%) of inter-myocyte fat (20). The procedures used for PI3-kinase assay, immunoprecipitation, and immunoblotting have been described previously (21). Representative data from one of three independent experiments are shown.

Lipid Metabolism and Measurement of Tissue TG Content—The measurements of [^{14}C]CO_2 production from [1-^{14}C]palmitic acid and lipogenesis from [1-^{14}C]acetate were performed using liver, muscle, and WAT slices, as described (18, 22). Liver and muscle homogenates were extracted, and their TG content was determined as described previously (18), with some modifications.

RESULTS

TZD Improve Insulin Resistance At the Expense of Obesity, whereas Heterozygous PPARγ Deficiency Improves Both Insulin Resistance and Obesity—To explain how insulin resistance could be improved by two opposite PPARγ activity states, supraphysiological activation of PPARγ and moderate reduction, we studied the phenotypes of untreated or PPARγ agonist-treated wild-type mice and untreated heterozygous PPARγ-deficient mice. We assessed PPARγ activity *in vivo* by measuring expression levels of lipoprotein lipase (23), fatty-acid translocase (FAT)/CD36 (24), and adipocyte fatty acid-binding protein/adipocyte P2 (25) (Fig. 1*A*), whose promoters contain peroxisome proliferator response element, in WAT, where PPARγ is expressed most predominantly *in vivo*. As expected, rosiglitazone-treated wild-type mice exhibited a significant increase in PPARγ activity as compared with untreated wild-type mice (Fig. 1*A*, *lanes 1* and *2*), whereas untreated heterozygous PPARγ-deficient mice showed a moderate decrease in PPARγ activity (Fig. 1*A*, *lanes 1* and *3*).

Untreated wild-type mice on the HF diet gained significantly more body weight than the mice on the HC diet (data not shown). Administration of rosiglitazone to wild-type mice increased significantly more body weight than vehicle on the HF diet (Fig. 1*B*, *lanes 1* and *2*). In contrast, heterozygous PPARγ deficiency reduced the increase in body weight on the HF diet (Fig. 1*B*, *lanes 2* and *3*). Treatment of wild-type mice with rosiglitazone significantly increased WAT mass (Fig. 1*C*, *lanes 1* and *2*), whereas untreated heterozygous PPARγ-deficient mice were protected from HF diet-induced increase in WAT mass (Fig. 1*C*, *lanes 2* and *3*). These data suggested that PPARγ determines the adiposity in proportion to its activity.

Treatment of wild-type mice with rosiglitazone improved hyperglycemia (Fig. 1*D*, *lanes 1*) and hyperinsulinemia (Fig. 1*E*, lane 1) on the HF diet as compared with untreated wild-type mice (Fig. 1, *D* and *E*, *lane 2*). Untreated heterozygous PPARγ-deficient mice were also protected from HF diet-induced hyperglycemia (Fig. 1*D*, *lanes 2* and *3*) and hyperinsulinemia (Fig. 1*E*, *lanes 2* and *3*). These findings indicate that TZD improve insulin sensitivity at the expense of obesity, whereas moderate reduction of PPARγ activity has potential as anti-obesity and anti-diabetic drugs.

Heterozygous PPARγ Deficiency Exerts Its Anti-obesity and Anti-diabetic Effects in Part through Leptin-dependent Pathways—The rectal temperature was lower in rosiglitazone-treated wild-type mice than that in untreated wild-type mice (Fig. 2*A*, *lanes 1* and *2*); on the contrary, it was significantly higher in untreated heterozygous PPARγ-deficient mice (Fig. 2*A*, *lanes 2* and *3*). The serum leptin (26) levels were slightly but not significantly lower in rosiglitazone-treated wild-type mice than those of untreated wild-type mice (Fig. 2*B*, *lanes 1* and *2*), whereas they were significantly higher in untreated heterozygous PPARγ-deficient mice (Fig. 2*B*, *lanes 2* and *3*). Thus the serum leptin levels were parallel to the rectal temperature. It was also noted that serum leptin levels were negatively correlated with PPARγ activity, suggesting that the serum leptin levels were parallel to the repression of leptin gene transcription by PPARγ/RXR (27). Moreover, leptin sensitivity as assessed by reductions in food intake and body weight change in response to exogenously administered leptin was significantly increased in heterozygous PPARγ-deficient mice as compared with wild-type mice on the HF diet (Fig. 2*C* and *D*, *lanes 3–6*). The degree of change in body weight induced by leptin treatment differed significantly ($p < 0.01$) between wild-type (−0.67 ± 0.09%) and heterozygous PPARγ-deficient

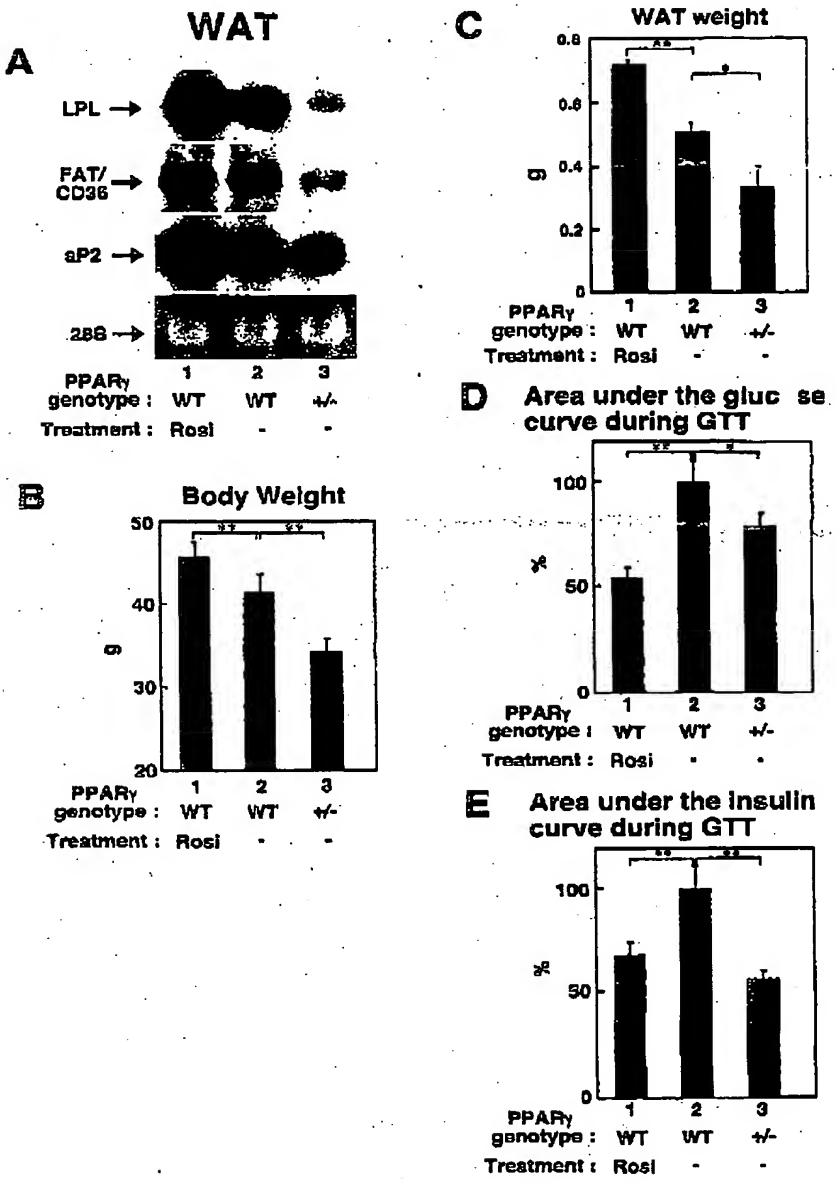

FIG. 1. **TZD improve insulin resistance at the expense of obesity, whereas heterozygous PPARγ deficiency improves both insulin resistance and obesity.** Amounts of the mRNAs of lipoprotein lipase (*LPL*), fatty-acid translocase (*FAT*)/CD36, adipocyte fatty acid binding protein/adipocyte P2 (*aP2*) in white adipose tissue (*A*), body weight (*B*), WAT weight (*C*), the values of area under the glucose curve (*D*), and area under the insulin curve (*E*) during glucose tolerance test (*GTT*) of wild-type (*WT*) and heterozygous PPARγ-deficient mice (+/−) untreated (−) or treated with rosiglitazone (*Rosi*) for 4 weeks (*A–E*) while on the high fat diet are shown. Rosiglitazone was given as a 0.01% food admixture. Each *bar* represents the mean ± S.E. (n = 5–10). *, $p < 0.05$; **, $p < 0.01$.

mice (−1.38 ± 0.11%). These data raised the possibility that increased leptin effects may contribute to the effects of the heterozygous PPARγ deficiency.

Heterozygous PPARγ Deficiency Decreases Lipogenesis in WAT, whereas TZD Stimulate Adipocyte Differentiation and Apoptosis, Thereby Both Preventing Adipocyte Hypertrophy—In WAT from untreated heterozygous PPARγ-deficient mice, expressions of lipoprotein lipase and CD36 were reduced, which may contribute to decreased TG content. In addition, expressions of lipogenic enzymes such as sterol regulatory element-binding protein (SREBP) 1c and SCD (stearoyl-CoA desaturase) 1 were reduced, and lipid synthesis was indeed significantly decreased in WAT from heterozygous PPARγ-deficient mice as compared with that in wild-type mice on the HF diet (Fig. 2*F*). Expression of β_3-adrenergic receptor (Fig. 2*E*, *lanes 2* and *3*) was increased presumably due to their increased leptin effects (Fig. 2, *B–D*) (28) and decreased PPARγ/RXR effects (29), and fatty acid oxidation was increased (data not shown). Decreased lipid synthesis and increased fatty acid oxidation as well as presumably decreased fatty acid influx in heterozygous PPARγ-deficient mice may in concert prevent adipocyte hypertrophy (Fig. 3*A*), and therefore obesity (Fig. 1, *B* and *C*, *lanes 2* and *3*), on the HF diet.

Interestingly, supraphysiological activation of PPARγ significantly reduced the average size of adipocytes under the HF diet (Fig. 3*A*, *lane 1* and *2*) as a result of a marked increase in

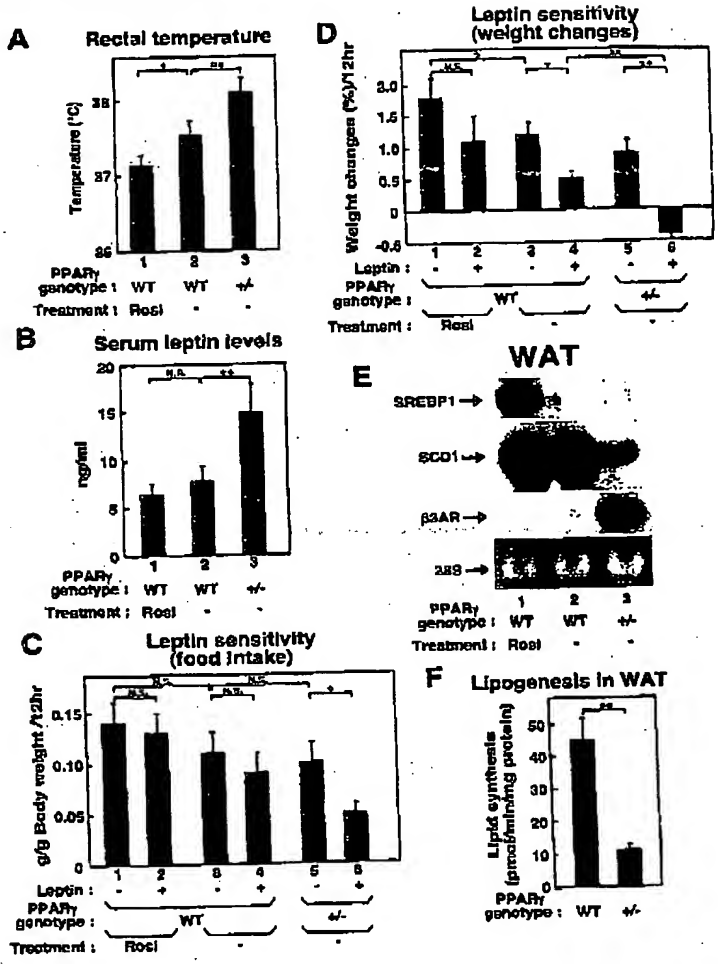

FIG. 2. **Heterozygous PPARγ deficiency improves both insulin resistance and obesity in part through leptin-dependent pathways.** The rectal temperature (*A*), serum leptin levels (*B*), amounts of the mRNAs of SREBP 1, stearoyl Co-A desaturase (*SCD*) 1, and β_3-adrenergic receptor (*AR*) in WAT (*E*), lipid synthesis from [^{14}C]acetate in WAT (*F*), effects of intraperitoneal leptin administration in wild-type (*WT*), and heterozygous PPARγ-deficient mice (+/−) untreated (−) or treated with rosiglitazone (*Rosi*) for 10 days (*B*) or 4 weeks (*A* and *C–F*) while on the HF diet are shown. Mice received an intraperitoneal injection of either leptin (10 μg/g/day) (+) or isotonic sodium chloride solution (−). Food intake/12 h (*left*) and weight changes/12 h (*right*) were measured. Rosiglitazone was given as a 0.01% food admixture. Each *bar* represents the mean ± S.E. (n = 5–10). *, $p < 0.05$; **, $p < 0.01$; *N.S.*, no significant difference; compared with untreated wild-type mice.

the number of newly differentiated small adipocytes and significant decrease in the number of large adipocytes with a concomitant induction of apoptosis of adipocyte (Fig. *3B*, *lanes 1* and *2*, and Fig. *3C*) (8). On the other hand, heterozygous PPARγ deficiency appeared to prevent HF diet-induced adipocyte hypertrophy without a significant change in the total number of adipocytes (Fig. *3B*, *lanes 2* and *3* and Fig. *3C*). These data suggest that the heterozygous PPARγ deficiency decreases lipogenesis in WAT, whereas TZD stimulate adipocyte differentiation and apoptosis, thereby both preventing adipocyte hypertrophy.

Adipocyte Hypertrophy Is Associated with Insulin Resistance—We next attempted to experimentally clarify the relationships between adipocyte hypertrophy and insulin resistance. To this end, we induced adipocyte hypertrophy by high fat feeding, leptin receptor deficiency, or agouti overexpression. The size of the adipose cells and the insulin resistance were increased in mice on a HF diet compared with those in mice on a HC diet (Fig. *3D*). The size of the adipose cells and the insulin resistance of db/db mice were also increased compared with their wild-type controls on both the HC and HF diet (Fig. *3D*). We obtained essentially similar results by using KKAy mice and their wild-type controls (KK) (Fig. *3E*). These findings support a close correlation between adipocyte hypertrophy and insulin resistance, even though a cause and effect relationship is again unproven. In this context, protection from adipocyte hypertrophy due to decreased lipid synthesis in WAT from heterozygous PPARγ-deficient mice (Fig. *2F*) may cause an increase in insulin sensitivity (Fig. *3F*).

Protection from Adipocyte Hypertrophy May Finally Lead to Alleviation of Insulin Resistance Presumably via a Decrease in Molecules Causing Insulin Resistance and Up-regulation of Insulin-sensitising Hormones—We tried to clarify the molecular link between adipocyte hypertrophy and insulin resistance. We examined the levels of expression of molecules secreted by WAT that regulate insulin sensitivity under the following four different conditions: HC feeding, HF feeding, HF feeding with heterozygous PPARγ deficiency, and HF feeding with PPARγ agonist. The HF diet significantly increased adipocyte size and at the same time increased the molecules causing insulin resistance, such as FFA and TNFα (Fig. *3*, *G* and *H*), and decreased the molecules causing insulin sensitivity, such as adiponectin (Fig. *3I*), in mice that exhibited insulin resistance as compared with mice on the HC diet (Fig. 1, *D* and *E*, *lanes 1* and *2*). (Replenishment of adiponectin in KKAy mice on the HF diet partially reverses insulin resistance even at the doses that do not significantly change adipocyte size (17).) In addition, treatment of wild-type mice with the PPARγ agonist rosiglitazone

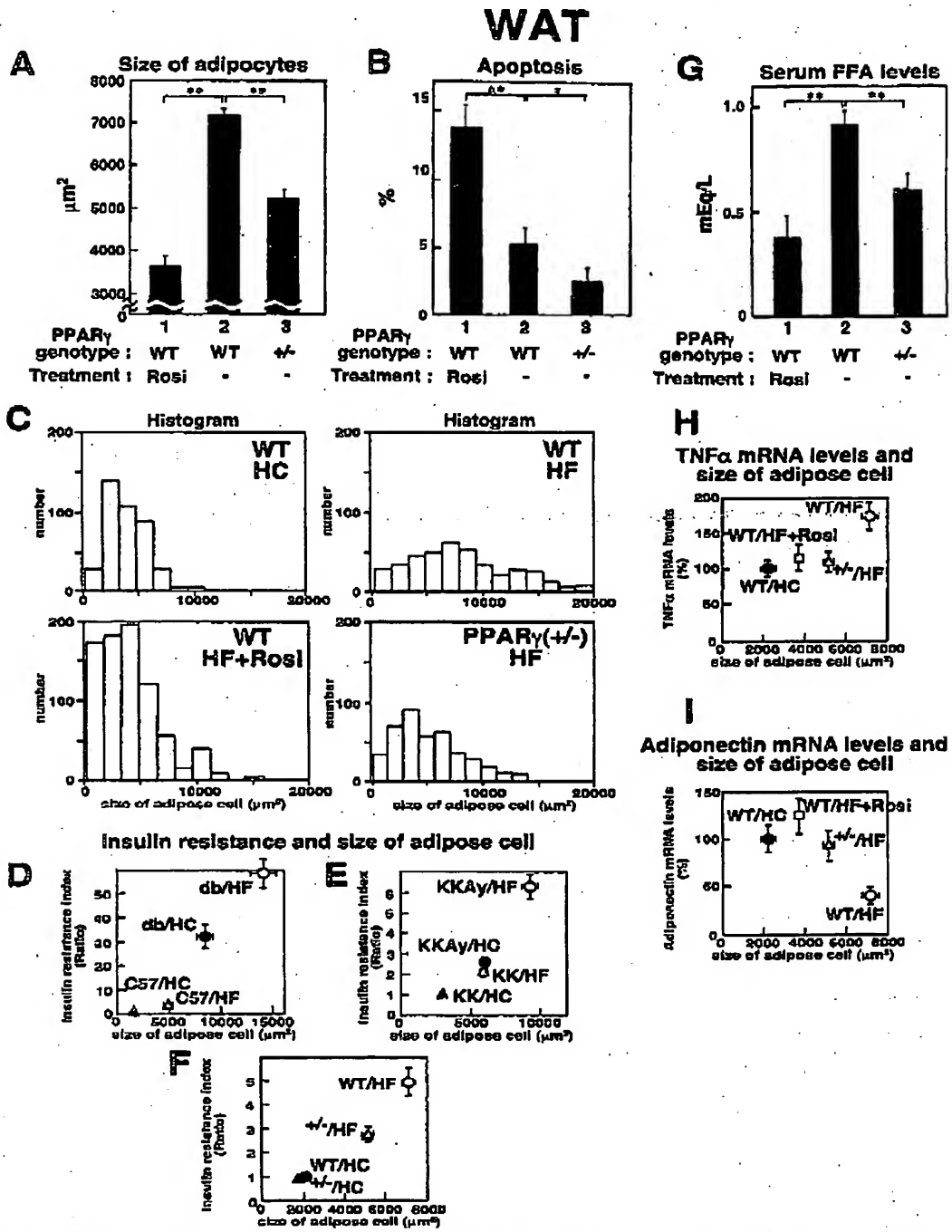

FIG. 3. **Both heterozygous PPARγ deficiency and TZD prevent adipocyte hypertrophy, which finally lead to alleviation of insulin resistance via a decrease in molecules causing insulin resistance and up-regulation of insulin-sensitising hormone, at least in part.** *A–E*, the average size of adipocytes (*A*), the ratio of apoptotic nuclei (*B*), distribution of adipose cell size (*C*) of epididymal WAT, the relationship between insulin resistance and average size of adipose cells (*D–F*), serum free fatty acid levels (*G*), the relationship between average size of adipose cells and amounts of the mRNAs of TNFα (*H*), or adiponectin (*I*) in WAT in wild-type (*WT*) and heterozygous PPARγ-deficient mice (+/−) (*A-C* and *F-I*) or C57 and db/db (db) mice (*D*) or KK and KKAy mice (*E*) untreated (−) or treated with rosiglitazone (*Rosi*) for 4 weeks while on the HC (*C–F*) or HF diet (*A–I*). Rosiglitazone was given as a 0.01% food admixture. Each *bar* represents the mean ± S.E. (n = 5–10). *, $p < 0.05$; **, $p < 0.01$; compared with untreated wild-type mice.

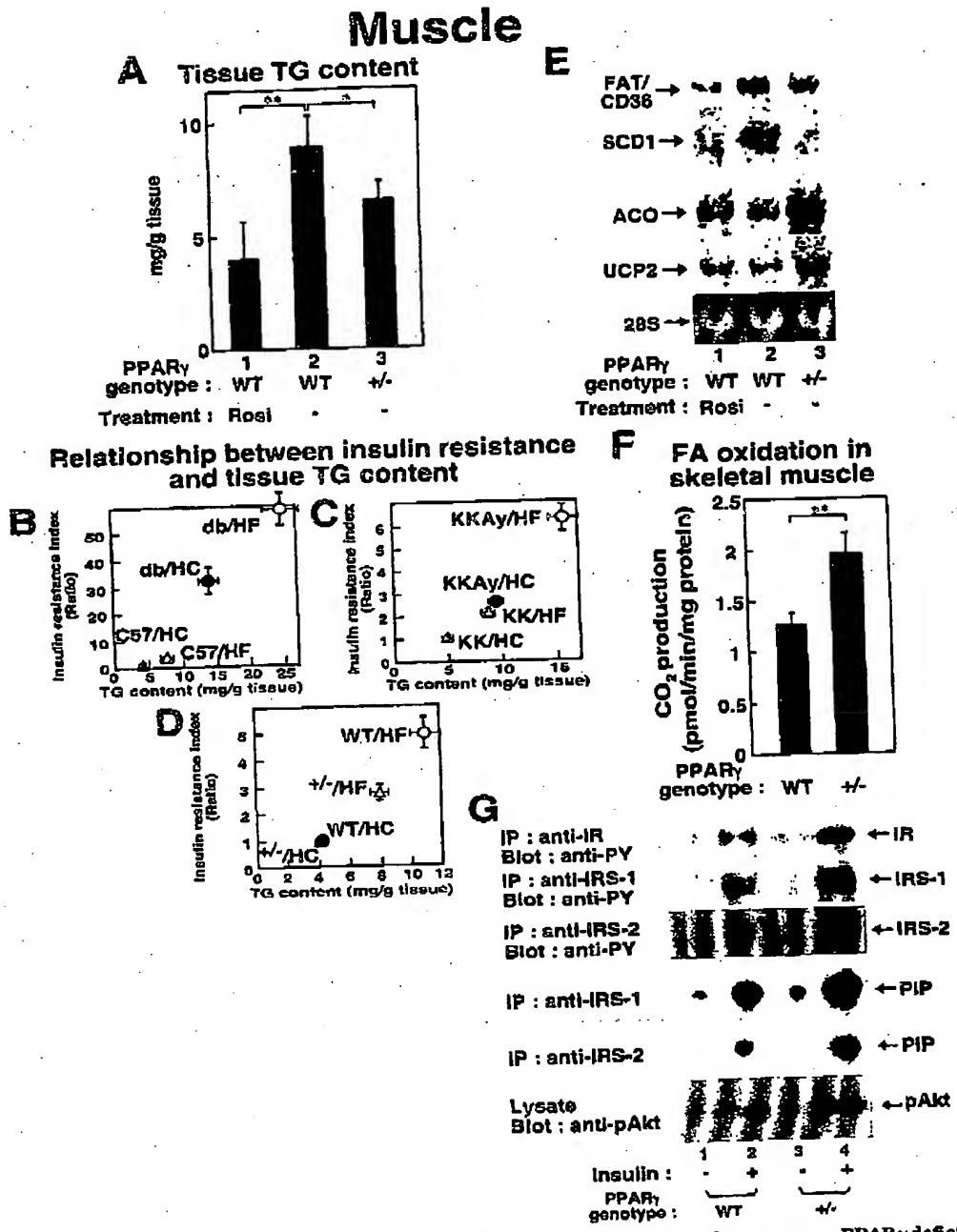

FIG. 4. **TZD indirectly decreases molecules involved in FFA influx into muscle, whereas heterozygous PPARγ deficiency increases fatty acid oxidation and molecules involved in energy dissipation, thereby both decreasing tissue TG content in muscle.** Tissue triglyceride (*TG*) content in skeletal muscle (*A*), the relationship between insulin resistance and tissue TG content in muscle (*B–D*), amounts of the mRNAs of fatty-acid translocase (*FAT*)/CD36, stearoyl Co-A desaturase (*SCD*) 1, acyl-CoA oxidase (*ACO*), and uncoupling protein (*UCP*) 2 (*E*), fatty acid (*FA*) oxidation (*F*), and insulin-induced tyrosine phosphorylation of insulin receptor (*IR*) and IRS-1 and -2, and insulin-stimulated PI3-kinase activity and insulin-induced phosphorylation of Akt (*G*) in muscle of wild-type (*WT*) and heterozygous PPARγ-deficient mice (*+/−*) (*A* and *D–G*) or C57 and db/db (*db*) mice (*B*) or KK and KKAy mice (*C*) untreated (*−*) or treated with rosiglitazone (*Rosi*) for 4 weeks while on the HF diet (*A–G*) or HC diet (*B–D*). Rosiglitazone was given as a 0.01% food admixture. Fatty acid oxidation was assessed by the measurements of $[^{14}C]CO_2$ production from [^{14}C]palmitic acid (*F*). Mice were stimulated with or without 1 μg/g body weight of insulin for 2 min. Lysates were immunoprecipitated (*IP*) with the antibodies indicated, followed by immunoblotting with the antibodies indicated or kinase assay for PI. Labeled PI (*PIP*) was subjected to thin layer chromatography and autoradiography as described previously (20) (*G*). Each *bar* represents the mean ± S.E. (n = 5–10). *, $p < 0.05$; **, $p < 0.01$ compared with untreated wild-type mice.

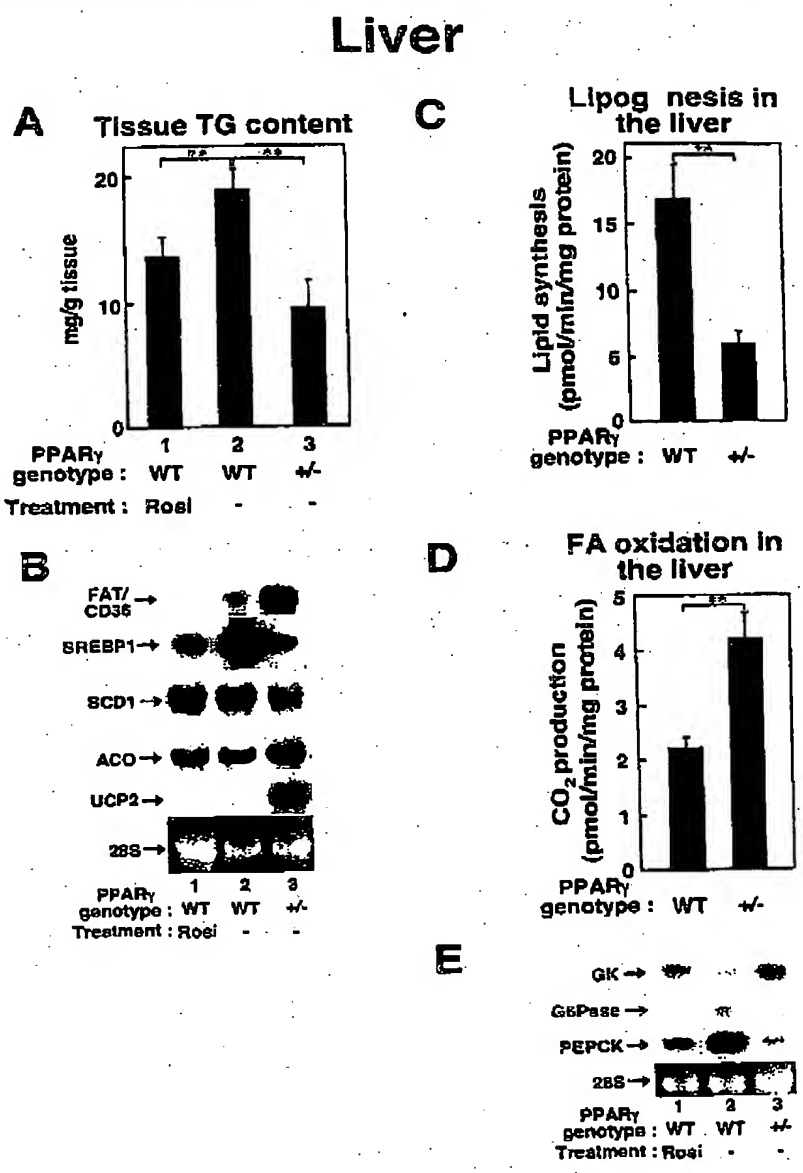

FIG. 5. **TZD indirectly decreases molecules involved in FFA influx into the liver, whereas heterozygous PPARγ deficiency combusts fatty acid and decreases lipogenesis, thereby both decreasing tissue TG content in liver.** Tissue triglyceride (*A*), amounts of the mRNAs of fatty-acid translocase (*FAT*/CD36, SREBP 1, stearoyl Co-A desaturase (*SCD*) 1, acyl-CoA oxidase (*ACO*), and uncoupling protein (*UCP*) 2 (*B*), lipid synthesis from [^{14}C]acetate in the liver (*C*), fatty acid (*FA*) oxidation (*D*), glucokinase, phosphoenolpyruvate carboxykinase (*PEPCK*) and glucose-6-phosphatase (*G6Pase*) (*E*), in liver of wild-type (*WT*) and heterozygous PPARγ-deficient mice (+/−) untreated (−) or treated with rosiglitazone (*Rosi*) for 4 weeks while on the HF diet. Rosiglitazone was given as a 0.01% food admixture. Fatty acid oxidation was assessed by the measurements of [^{14}C]CO_2 production from [^{14}C]palmitic acid (*D*). Each bar represents the mean ± S.E. (n = 5–10). *, $p < 0.05$; ⇒, $p < 0.01$; compared with untreated wild-type mice.

heterozygous PPARγ deficiency, both of which resulted in protection against HF diet-induced adipocyte hypertrophy, significantly decreased FFA and TNFα (Fig. 3, *G* and *H*) and increased adiponectin (Fig. 3*I*) and at the same time ameliorated insulin resistance (Fig. 1, *D* and *E*) on the HF diet. However, treatment of wild-type mice with a PPARγ agonist increased adipose tissue mass (Fig. 1*C*, *lane 1*) and body weight (Fig. 1*B*, *lane 1*), whereas heterozygous PPARγ deficiency significantly decreased them (Fig. 1, *B* and *C*, *lane 3*). These findings raised the possibility that levels of expression of molecules regulating insulin sensitivity may be more closely related to adipocyte size than PPARγ activity, adipose tissue mass, or body weight *in vivo*. Large adipocytes are known to be resistant to anti-lipolytic action of insulin, thereby releasing a large amount of FFA (80); however, the mechanisms underlying the correlation between larger adipocytes and up-regulation of TNFα and/or down-regulation of adiponectin remain to be elucidated.

TZD Indirectly Decrease Molecules Involved in Fatty Acid Influx into Muscle/Liver, whereas Heterozygous PPARγ Deficiency Increases Fatty Acid Combustion and Decreases Lipogenesis via Increased Leptin Effect, Thereby Both Decreasing Their Tissue TG Content—Interestingly, both supraphysiological activation of PPARγ and moderate reduction of PPARγ activity significantly reduced tissue TG content in muscle and liver (Fig. 4*A* and Fig. 5*A*), suggesting that insulin resistance has an excellent correlation with tissue TG content in muscle and liver (Fig. 1, *D* and *E*, Fig. 4*A*, and Fig. 5*A*) (15).

In wild-type mice treated with rosiglitazone, the decreased

tissue TG content in muscle/liver, where PPARγ was less abundantly expressed as compared with what was in WAT, was presumably via reduced expression of molecules involved in FFA influx into muscle/liver (Fig. 4*E* and Fig. 5*B*, *lanes 1* and *2*). On the other hand, heterozygous PPARγ deficiency reduced expression of lipogenic enzymes such as SCD1 (Fig. 4*E* and Fig. 5*B*, *lanes 2* and *3*) and SREBP 1 (Fig. 5*B*, *lanes 2* and *3*), and indeed significantly reduced lipogenesis (Fig. 5*C*), presumably due to increased leptin effects (Fig. 2, *B–D*) (28), may reduce tissue TG content in muscle/liver (Fig. 4*A* and Fig. 5*A*, *lanes 2* and *3*).

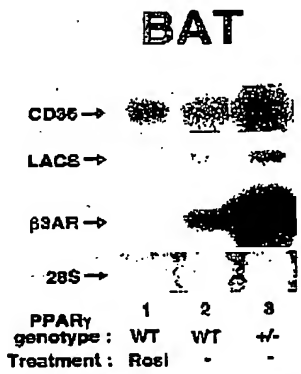

FIG. 6. **Heterozygous PPARγ deficiency increases expression of molecules involved in β-oxidation and β_3-adrenergic receptor in BAT.** Amounts of the mRNAs of fatty-acid translocase (*FAT*/CD36, long chain acyl-CoA synthetase (*LACS*), and β_3-adrenergic receptor (*AR*) in BAT of wild-type (*WT*) and heterozygous PPARγ-deficient mice (+/−) untreated (−) or treated with rosiglitazone (*Rosi*) for 4 weeks while on the high fat diet. Rosiglitazone was given as a 0.01% food admixture.

Heterozygous PPARγ Deficiency Increases Fatty Acid Combustion and Molecules Involved in Energy Dissipation via PPARα Pathways in Liver, Muscle, and BAT—Moreover, in muscle/liver from heterozygous PPARγ-deficient mice, increased expression of enzymes involved in β-oxidation such as acyl-CoA oxidase and that of molecules involved in energy dissipation such as UCP2 (Fig. 4*E* and Fig. 5*B*, *lanes 2* and *3*) were observed. Fatty acid oxidation was indeed significantly increased in muscle/liver from heterozygous PPARγ-deficient mice as compared with that in wild-type mice on the HF diet (Fig. 4*F* and Fig. 5*D*). These alterations may be an additional mechanism for reduced TG content in muscle/liver of heterozygous PPARγ-deficient mice. Since these effects were recapitulated by treatment of wild-type mice with Wy-14,643, a PPARα agonist as reported (18, 19, 31) (data not shown), PPARα pathways appeared to be activated in the liver of heterozygous PPARγ-deficient mice.

In the BAT, where PPARα was relatively abundantly expressed compared to that in WAT, significant increases in the expression of molecules involved in fatty acid combustion presumably via activation of PPARα pathways (18, 19, 31) and β_3-adrenergic receptor (Fig. 6), due to increased leptin effects (28) and decreased PPARγ effects (29), were observed. These alterations in concert may provide the mechanism that increased energy expenditure by heterozygous PPARγ deficiency (Fig. 2*A*).

Heterozygous PPARγ Deficiency Indeed Improves Insulin Signal Transduction and Insulin Actions in Each Target Organ—Increased tissue TG content has been reported to interfere with insulin-stimulated phosphatidylinositol (PI) 3-kinase activation and subsequent GLUT4 translocation and glucose uptake (15). Next, we tried to experimentally clarify the relationships between tissue TG content and insulin resistance. To do so, we increased tissue TG content by high fat feeding, leptin

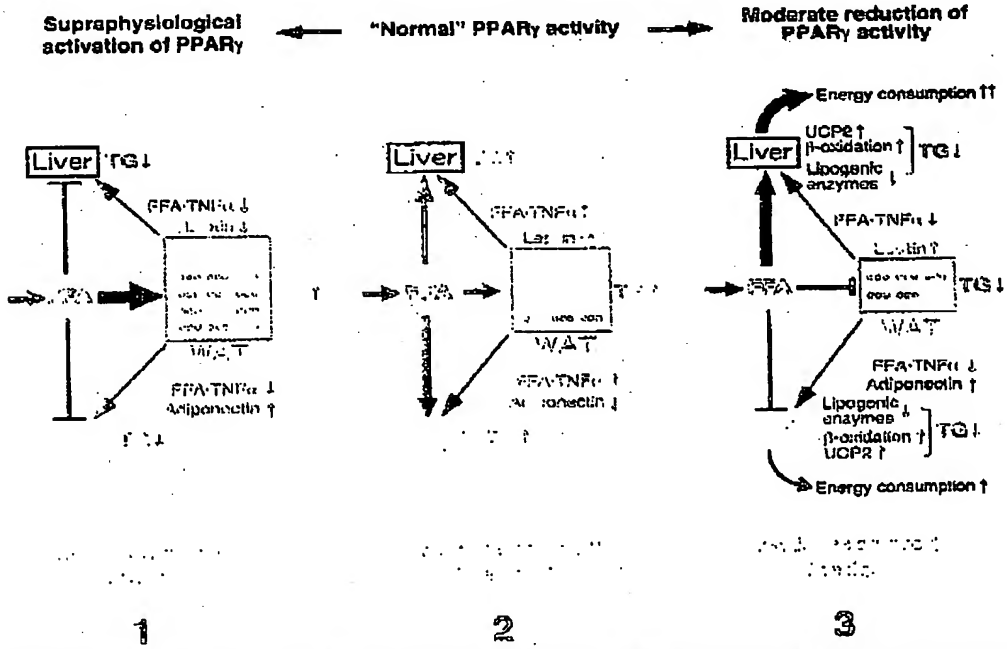

FIG. 7. **Proposed mechanisms for the regulation of insulin sensitivity and adiposity by PPARγ.** See "Both Heterozygous PPARγ Deficiency and PPARγ Agonist Improve Insulin Resistance Presumably Due to Decreased TG Content in Muscle/Liver as Well as Prevention of Adipocyte Hypertrophy" under "Discussion."

receptor deficiency, or agouti overexpression. The tissue TG content of skeletal muscle and insulin resistance were increased in mice on the HF diet compared with those in mice on the HC diet (Fig. 4*B*). The tissue TG content of skeletal muscle and insulin resistance of db/db mice were also increased compared with their wild-type controls on both the HC and HF diets (Fig. 4*B*). We obtained essentially similar results by using KKAy mice and their wild-type controls (KK) (Fig. 4*C*). These findings raise the possibility that increases in tissue TG content are associated with insulin resistance. Conversely, decreased tissue TG content due to decreased lipid synthesis and increased fatty acid oxidation in muscle/liver from heterozygous PPARγ-deficient mice (Fig. 4*F* and Fig. 5, *C* and *D*) may cause an increase in insulin sensitivity (Fig. 4*D*). Shulman and co-workers (32) proposed a cause and effect relationship between the accumulation of intracellular fatty acid-derived metabolites and insulin resistance. However, there are instances in which tissue TG content actually does not change in another scenario that also causes insulin resistance, *i.e.* adipose-selective targeting of the GLUT4 gene (32). Thus, interpretation should be done with caution, and decreased tissue TG content in muscle/liver is one possible mechanism for the results of increased insulin sensitivity in heterozygous PPARγ-deficient mice.

Consistent with this possibility, decreased TG content in muscle of heterozygous PPARγ-deficient mice indeed improved insulin signal transduction in muscle, as demonstrated by increases in insulin-induced tyrosine phosphorylation of insulin receptor, insulin receptor substrate (IRS)-1 and IRS-2, and insulin-stimulated PI3-kinase activity in phosphotyrosine, IRS-1 and IRS-2 immunoprecipitates, and insulin-stimulated Akt activity in skeletal muscle (Fig. 4*G*). The reduction of TG content in liver of heterozygous PPARγ-deficient mice was associated with increased expression of glucokinase and decreased expression of enzymes involved in gluconeogenesis such as phosphoenolpyruvate carboxykinase and glucose-6-phosphatase (Fig. 5*E*), indicating increased insulin actions also in liver.

DISCUSSION

Both Heterozygous PPARγ Deficiency and PPARγ Agonist Improve Insulin Resistance Presumably Due to Decreased TG Content in Muscle/Liver as Well as Prevention of Adipocyte Hypertrophy—We attempted to explain how insulin resistance could be improved by the following two opposite PPARγ activity states: a potent activation of PPARγ and its moderate reduction. We did so by using heterozygous PPARγ-deficient mice and a pharmacological activator of PPARγ in wild-type mice. On the basis of experimental results obtained in this study, we propose the following hypothesis on the mechanisms for the regulation of insulin sensitivity by PPARγ (Fig. 7).

As shown in the Fig. 7, *panel 2*, on the HF diet, "normal" amounts of PPARγ activity seen in wild-type mice increase TG content in WAT, skeletal muscle, and liver due to a combination of increased fatty acid influx into WAT, skeletal muscle, and liver and HF diet-induced leptin resistance, leading to insulin resistance and obesity. Moreover, hypertrophic adipocytes may increase the secretion of molecules causing insulin resistance, such as FFA (15) and TNFα (16), and decrease that of an insulin-sensitizing hormone, such as adiponectin (17).

As shown in Fig. 7, *panel 1*, supraphysiological activation of PPARγ way beyond that by TZD stimulates adipogenesis, which promotes a flux of FFA from liver and muscle into WAT, leading to a decrease in TG content in liver and muscle and improvement of insulin sensitivity at the expense of increased WAT mass, *i.e.* obesity. Moreover, TZD induce adipocyte differentiation and apoptosis, thereby increasing the number of small adipocytes, which finally lead to alleviation of insulin resistance presumably via a decrease in molecules causing insulin resistance, such as FFA and TNFα, and up-regulation of insulin-sensitizing hormone adiponectin, at least in part.

By contrast, as shown in the Fig. 7, *panel 3*, moderate reduction of PPARγ activity observed in untreated heterozygous PPARγ-deficient mice decreases TG content in WAT, skeletal muscle, and liver. This effect is due to a combination of increased leptin expression by antagonism of PPARγ-mediated suppression of the gene, thereby reducing expression of lipogenic enzymes, and consequent activation of PPARα pathway in liver, BAT, and skeletal muscle, leading to an increase in expression of UCP2 and enzymes involved in β-oxidation. These observations fit well with the recently demonstrated effects of PPARα agonists on insulin resistance (33) and decreased fatty acid combustion in PPARα-deficient mice (34). Moreover, direct antagonism of PPARγ to reduce lipogenesis in WAT prevents adipocyte hypertrophy under the HF diet, thereby reducing the molecules causing insulin resistance, such as FFA and TNFα, and up-regulating the insulin-sensitising hormone adiponectin, at least in part. These alterations lead to prevention against HF diet-induced obesity and insulin resistance. The data showing that moderate reduction of PPARγ activity resulted in increased insulin sensitivity were further confirmed by the observation that treatment of heterozygous PPARγ-deficient mice with a low dose of TZD caused the re-emergence of insulin resistance (9).

This study has thus revealed the mechanisms whereby both PPARγ agonist and heterozygous PPARγ deficiency have a similar effect on insulin sensitivity. However, it should also be noted that PPARγ agonist and heterozygous PPARγ deficiency have an opposite effect on adiposity and energy expenditure which appear to be more directly regulated by PPARγ activity.

Both Heterozygous PPARγ Deficiency and TZD Prevent Adipocyte Hypertrophy via Different Mechanisms, Thereby Finally Contributing to Increased Insulin Sensitivity—Although both heterozygous PPARγ deficiency and PPARγ agonist finally improve insulin resistance via decreased TG content in muscle/liver and prevention of adipocyte hypertrophy, there are some important differences between them. First, although both reduced TG content in muscle/liver, heterozygous PPARγ deficiency did so via activation of fatty acid combustion and energy dissipation, whereas TZD did so via potent stimulation of adipogenesis, thereby increasing fatty acid flux from muscle/liver into WAT. Second, both prevented HF diet-induced adipocyte hypertrophy, and TZD markedly increased the number of newly differentiated small adipocytes, whereas heterozygous PPARγ deficiency appeared not to change the total number of adipocytes.

Taken together, all of these differences are consistent with the notion that activation of PPARγ plays a role in energy storage and adiposity, and reduction of PPARγ causes energy dissipation and prevention of adiposity.

In conclusion, although by different mechanisms, both heterozygous PPARγ deficiency and PPARγ agonist improve insulin resistance via decreased TG content in muscle/liver and prevention of adipocyte hypertrophy (Fig. 7).

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